KATHOLIEKE UNIVERSITEIT
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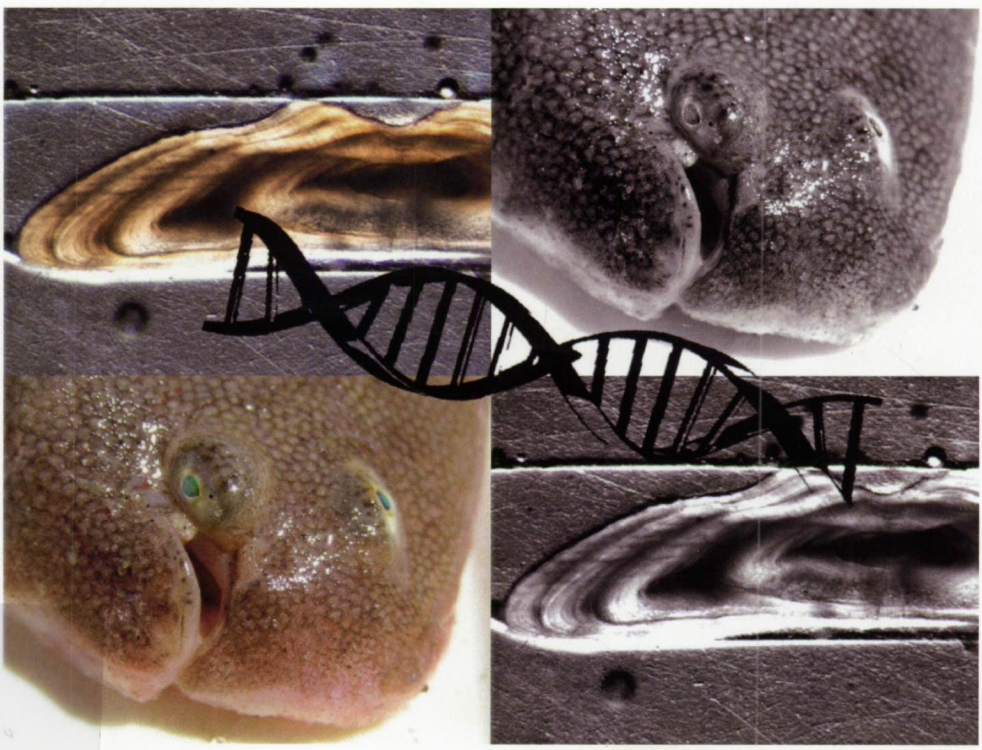
Faculty of Science
Department of Biology

CONNECTIVITY AND GENETIC STABILITY IN SOLE
(SOLEA SOLEA)

Els CUVELIERS



Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Science (Biology)

January 2011

Katholieke Universiteit Leuven
Faculteit Wetenschappen
Departement Biologie
Laboratorium voor Diversiteit en Systematiek van Dieren

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CONNECTIVITEIT EN GENETISCHE STABILITEIT BIJ TONG
(SOLEA SOLEA)

Els Cuveliers

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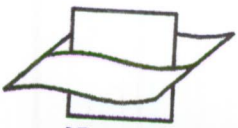
Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Wetenschappen

Januari 2011



ISBN 978-90-8649-389-0
D/2011/10.705/7

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Het onderzoek voorgesteld in dit proefschrift werd gesteund door een beurs verleend door het Agentschap voor Innovatie door Wetenschap en Technologie (IWT-Vlaanderen).

DANKWOORD

Tijdens de voorbije vier jaar van mijn doctoraat heb ik veel nieuwe dingen geleerd, mooie plaatsen ontdekt en interessante mensen ontmoet. Zonder de hulp en steun van zoveel mensen om me heen was het mij nooit gelukt. Daarom wil ik hier graag bepaalde mensen in het bijzonder bedanken.

Mijn promotor Prof. Dr. Filip Volckaert wil ik graag bedanken om mij de kans te geven een doctoraat te maken op een marien onderwerp in zijn groep en daarnaast ook mee te draaien in andere projecten. Naast alle steun en positivisme apprecieer ik ook de menselijke manier waarop je je groep leidt.

Mijn co-promotor Dr. Gregory Maes. Vaak heb ik gevloekt op de commentaren die je steeds had, de nieuwe software die je ontdekt had en die ik toch ook nog eens moest uitproberen, of de extra taakjes die je voor mij in petto had. Toch besef ik dat ik er zeer veel uit geleerd heb. Ik ben je zeer dankbaar voor je onuitputtelijke en aanstekelijke enthousiasme voor het vak, de interessante ideeën en ophelderende (of soms net verwarrende?) discussies. Ook voor de vele 'peptalk' en spreekwoordelijke schouderklopjes, bedankt!

I would like to thank all other members of my examination committee for providing me with useful comments for improving my thesis: Dr. Dorte Bekkevold, Prof. Dr. Olivier Honnay and Prof. Dr. Steven Bouillon. Prof. Dr. Adriaan Rijnsdorp, bedankt voor alle input en interesse die je toonde tijdens het opvolgen van mijn werk. Prof. Dr. Em. Frans Ollevier, ik vond het fijn dat u na mijn licentiaatsthesis ook mijn doctoraat nog wilde beoordelen.

Prof. Dr. Audrey Geffen, thank you for learning me more about the wonderful world of otoliths and for providing me (and Jef) the opportunity to come to Norway to analyze my samples in a beautiful surrounding.

I would like to thank Dr. Geneviève Lacroix from the MUMM for the nice collaboration and for sharing some interesting results on the hydrodynamic model.

Mijn thesisstudenten, Stefanie en Charlotte en ook Sarah G. en Bart héél véél dank voor alle hulp met het labowerk. Meteen wil ik ook alle mensen bedanken die me hebben geholpen met het vangen van tong, de ene keer in al wat betere weersomstandigheden dan de andere, de ene gezegend met betere zeebenen dan de andere: Jef, Sarah G., Sara R., Stefanie, Kim, Eveline, Filip,...Ook de bemanning van de Zeeleeuw, de Belgica en de Broodwinner, en het VLIZ wil ik bedanken voor de jarenlange logistieke ondersteuning.

Vervolgens wil ik alle collega's van het labo bedanken voor de toffe sfeer en de leuke babbels. Het is zeer uniek om met zoveel fijne mensen samen te werken (of koffie te drinken ☺). Veel dank aan alle ATP'ers en in het bijzonder Conny, Ria en Eddy die reeds vanaf het begin van mijn doctoraat paraat stonden voor logistieke of administratieve hulp en zonder wie het labo vierkant zou draaien.

Vooral een speciaal woordje van dank en dikke knuffel voor mijn naaste collega's van het eerste verdiep. In het begin waren er Griet, Anton, Jef, Maarten (aka 'Muso'), Maarten S. en Erika. Nadien kwamen Fre, Dirk en Nellie erbij om hun plaats in te nemen op de eilanden en het waren volwaardige vervangers!! Ook Maarten V., de Westvlaamse meisjes Sara en Eveline, bedankt voor de toffe babbels. Als jullie binnen enkele jaren nood hebben aan wat aanmoedigende woorden of ontspanning, zijn jullie altijd welkom! Verder ook nog Tine, Alessia, Bart, Alex^2, Dorien, Jo-Ann, Jeroen, Auguste,... kortom iedereen die de genetica lunches, diners, enzovoort zeer aangenaam maakten en altijd klaar stonden om mij op één of andere manier te helpen. 'S.O.S Joost' ☺, ook voor jou een eervolle vermelding en vooral nen dikke merci voor de statistische hulp en verhelderende ideeën.

Mijn vrienden die steeds klaarstonden voor een ontspannende activiteit tussendoor, maar die ik de laatste tijd nogal heb verwaarloosd. Naar het einde toe wordt een doctoraatsstudent steeds asocialer, dat hebben jullie vast gemerkt...Ik hoop er gauw verandering in te brengen!!!

Mijn grootste dank gaat echter uit naar mijn familie en in het bijzonder mijn ouders. Mama en papa, ik ben jullie zeer dankbaar voor alle kansen die jullie mij steeds hebben gegeven. Jullie hebben mij steeds gesteund, aangemoedigd en altijd interesse getoond voor wat ik deed. Bedankt hiervoor! Ook mijn twee schatten van zussen, schoonbroer en nichtje die ik voor geen geld van de wereld zou kunnen missen, bedankt voor jullie steun! Eef, ook erg bedankt voor het maken van de tekening.

Jef, jou wil ik speciaal bedanken. Niet alleen voor alle hulp bij de staalnames, met de otolieten in Noorwegen, voor de nuttige discussies, je hulp als co-auteur en het nalezen van mijn teksten (niet iedereen kan zeggen dat zijn lief niet alleen doet alsof hij geïnteresseerd is in otolieten en vissen, maar het ook werkelijk is). Vooral voor alle steun en motivatie die ik van jou heb gekregen. Voor je eindeloze geduld (!) en begrip! Ik kijk er naar uit om samen aan een nieuw hoofdstuk te beginnen; het wordt er zeker één met een hoge impactfactor ☺.

Els

LIST OF ABBREVIATIONS

AMOVA	Analysis of molecular variance
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Ad_j	Juvenile area of the adult fish
AIc	Assignment Index
A/J	Adult/Juvenile
AR	Allelic Richness
bp	base pair
C	Core
CI	Confidence Interval
Circ	Circularity
Conc	Concentration
Cyt *b*	Cytochrome *b*
E	Edge
EDTA	Ethylene Diamine Tetra-acetic Acid
EFD	Elliptic Fourier Descriptors
Ellipt	Ellipticity
F	Fishing mortality
FAO	Food and Agriculture Organisation of the United Nations
FCA	Factorial Correspondence Analysis
FD	Feret Diameter
Fpa	Precautionary approach fishing mortality (reference point)
F_{IS}	Inbreeding coefficient
F_{ST}	Fixation index
Hd	Haplotype diversity
He	Unbiased expected heterozygosity
Ho	Observed heterozygosity
HWE	Hardy Weinberg Equilibrium
IBD	Isolation By Distance
ICES	International Council for the Exploration of the Sea
ICPMS	Inductively Coupled Plasma Mass Spectrometry
K	Number of clusters
kya	Thousand years ago
LA-ICPMS	Laser ablation- Inductively Coupled Plasma Mass Spectrometry
LDFA	Linear Discriminant Function Analysis
LD	Linkage Disequilibrium
LOD	Limit of Detection
MANOVA	Multiple Analysis of Variance
MDS	Multi-dimensional scaling analysis
MLE	Maximum Likelihood Estimator
MNA	Mean Number of Alleles
mtDNA	Mitochondrial DNA
NA	Number of alleles
N_e	Effective population size

N_c	Census population size
NIES	National Institute for Environmental Studies, certified reference material no.22 (fish otolith)
OW	Otolith Weight
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
p	p-value
Rect	Rectangularity
RSD	Relative Standard Deviation
Round	Roundness
Sal	Salinity
SB-ICPMS	Solution-Based Inductively Coupled Plasma Mass Spectrometry
SDS	Sodium Dodecyl Sulphate
SMM	Stepwise Mutation Model
SL	Standard Length
SNP	Single Nucleotide Polymorphism
SRM	Standard Reference Material
SSB	Spawning Stock Biomass
Tg	Generation time (years)
TL	Total Length
v.	Version
W	Weight
Vk	Variance in reproductive success
vAlc	Variance of the assignment index

TABLE OF CONTENT

GENERAL INTRODUCTION

Marine fish stocks are declining in abundance due to a combination of environmental and anthropogenic threats such as global climate change, habitat degradation and overexploitation (Myers & Worm 2003; Mullon et al. 2005). In 2007, over 75 % of the monitored stocks were either fully exploited or overexploited (FAO 2008). Many marine fish are characterized by highly variable reproductive success and a type III survival with high mortality during early life (Hedgecock 1994), making them extremely vulnerable to environmental changes. The worldwide overfishing may increase the extinction risk of target and by-catch species even more (Hutchings 2000).

Information on population connectivity is crucial to understand the dynamics of fish populations and take proper management actions to conserve marine biodiversity. Recent recommendations from the European Union (European Fisheries and Aquaculture Research Organisation-EFARO) recognize the need for studies on the spatial and temporal population structure of exploited fish populations to improve current stock assessments and to predict the effects of environmental and anthropogenic changes. Furthermore, there is a need to address fisheries induced changes in the genetic composition of exploited fish stocks (Law 2007). The DNA analysis of long time series of archived material such as scales or otoliths is crucial to study such evolutionary changes.

In this study, we examine the connectivity and temporal genetic stability of sole (*Solea solea*) in the Northeast Atlantic Ocean, with a focus on the Southern North Sea as case study. The first part focuses on contemporary patterns of connectivity and population structure of sole. Two types of markers were used to address our research questions: neutral genetic markers and otolith microchemistry and shape. The second part of the thesis deals with temporal genetic stability of the species in the North Sea and is based on genetic markers.

The general introduction covers the concept of 'connectivity' in marine environments. The methods that are currently available to study connectivity are described, with special attention to the techniques used in this thesis. Second, some concepts in vogue in fisheries management and conservation are addressed and an introduction to our study system and overview on the state-of-the art of the research on *Solea solea* is presented. We conclude with the objectives of the study and the outline of the thesis.

1. Connectivity in marine ecosystems

Population connectivity can be defined as the extent to which marine populations in different parts of the species' range are connected by the exchange of larvae, juveniles or adults (Palumbi 2003). Movements in marine environments can occur over small or large distances and for several reasons. Some organisms move in order to find food, others to find a mating partner or to avoid unfavorable conditions. In either case, these movements of individuals make that habitats become more connected and that processes happening at one location may influence dynamics at another location (Gaines et al. 2007). Connectivity allows the colonization of empty habitats or the recolonization of extinct sites. Therefore, it plays a fundamental role in population dynamics, community structure and the resilience of populations against exploitation. Knowledge of connectivity is essential for a good understanding of ecosystem responses to changing environmental conditions (Gawarkiewicz et al. 2007). Also in fisheries management, connectivity plays an important role. First of all, for the delineation of appropriate management units and secondly, to identify the vulnerable areas that need to be protected from exploitation (Fogarty & Botsford 2007). Patterns of connectivity are determined by a combination of life history characteristics (e.g. fecundity, pelagic larval duration), behavior (e.g. spawning, dispersal characteristics) and hydrodynamic processes (e.g. retention zones, stratification of the water column) (Sponaugle et al. 2002).

1.1. Ecological models of dispersal

Knowledge on the dynamics of local subpopulations requires the inclusion of different life stages since migration or dispersal of individuals might occur at different levels: adults at spawning grounds, larval stages, and juveniles in nursery areas, (sub) adults at the feeding grounds or a combination of these. Classically, the migration triangle is used as a conceptual model in ecology linking ontogenetic life stages with specific habitats and demonstrating the role of migrations in connecting these habitats (Harden Jones 1968) (Fig. 1a). Many fish produce pelagic larvae which drift in the prevailing currents to suitable nursery grounds. There they will grow until they are large enough to swim to the feeding grounds. To complete their life cycle, adults need to migrate towards suitable spawning grounds. The original model of Harden Jones (1968) describes a closed circuit of migration with individuals

always returning to the place where they were spawned ('philopatry'). The member-vagrant theory of Sinclair (1988) expands on this theory and emphasizes the importance of life cycle closure (i.e. 'members') as a requirement for reproductive isolation (Fig. 1b). Individuals that deviate from the population's trajectory (i.e. 'vagrants') get lost to the population.

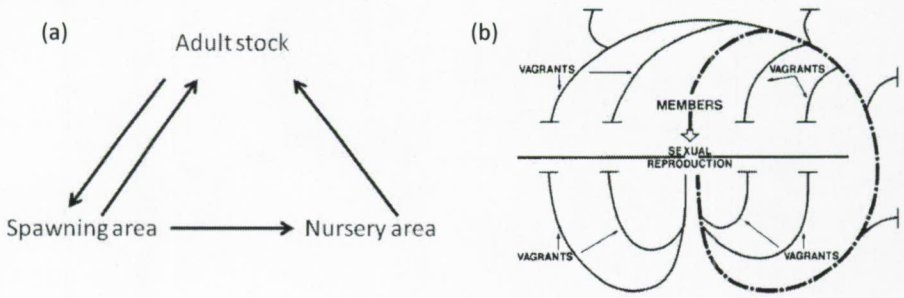

Fig. 1 (a) Migration triangle (Harden Jones 1968); (b) Member-vagrant hypothesis (Sinclair 1988)

According to Secor (1999) the migration circuit is a path that can expand or contract according to the energetic demands of a population (Fig. 2). Individuals might deviate from the mean trajectory in order to find more favorable habitats, but if they deviate too much they cannot rejoin the migration circuit (Secor 1999). Groups with different seasonal migration behaviors between feeding areas and spawning areas but sharing the same spawning ground might exist, resulting in multiple migration patterns within one population. Such groups are defined as 'contingents', as described by Clark (1968).

Since its original development and use in terrestrial ecology, the metapopulation concept (Levins 1970) has been increasingly integrated in marine systems and many of the assumptions of the classical definition have been relaxed to some degree (Wright et al. 2006). The number of patches may be finite, they can differ in size and extinction is not required (Kritzer & Sale 2004). A metapopulation refers to a system of discrete local subpopulations with their own local dynamics but with a large degree of connectivity and demographic influences from other local populations through dispersal of individuals (McQuinn 1997). Metapopulations differ from patchy populations, where individuals are also distributed among discrete groups but share one common larval pool with inter-population exchange so large that all populations equally affect each other (Kritzer & Sale 2004).

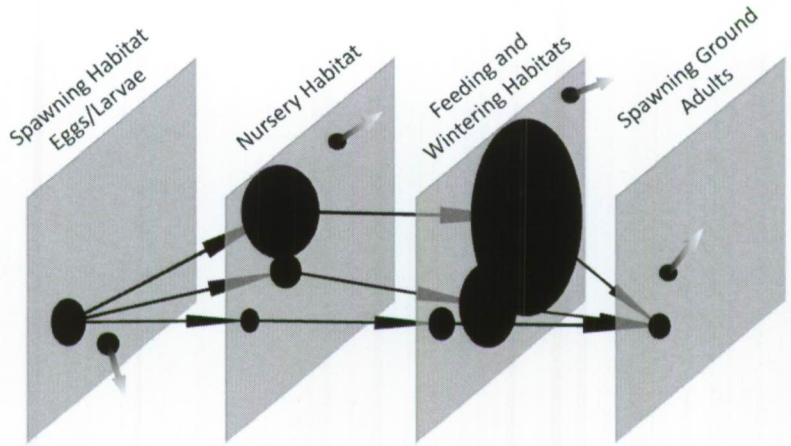

Fig. 2 Migration model after Secor (1999). Each pane is a cross-sectional profile of spatial occurrence (horizontal and vertical axes represent longitudes and latitude). Each line represents a different migration circuit while the centroids represent the dispersal pathway of the population aggregations. Open arrows indicate vagrants that possibly get lost.

1.2. Genetic population connectivity

- **Four evolutionary forces**

Genetic variation in populations is created, maintained or lost through four important processes: mutation, genetic drift, gene flow, and selection (Mills 2007). In general, mutations tend to occur relatively slowly and are only relevant from an evolutionary perspective (thousands of years). Genetic drift occurs when allele frequencies change randomly from one generation to the next, leading to the fixation of some alleles and the loss of others. Gene flow from one population to another neighboring population generally results in an increase in genetic variation, which increases the ability of a population to resist the negative effects of genetic drift. Too much gene flow on the other hand results in homogenization of allele frequencies among populations and might prevent local adaptation, unless selection coefficients are strong enough to create a genomic barrier. The relative importance of each process depends on the effective population size (see 1.3). In small populations, drift is more important; in larger populations, allelic diversity changes in response to natural selection.

- **Factors shaping population structure**

The level of observed population structure can be influenced by a range of factors, including migration, population size (N_e) and population history (Hauser & Carvalho 2008) (Fig. 3). Lack of allele frequency variance does not necessarily imply high connectivity but it can also reflect insufficient time since separation to establish a state of drift – migration equilibrium. Since most temperate marine fish occupy habitats only since the end of the last ice age (20 000 years ago), many genetic patterns are affected by the population history and many populations might not have reached migration-drift equilibrium.

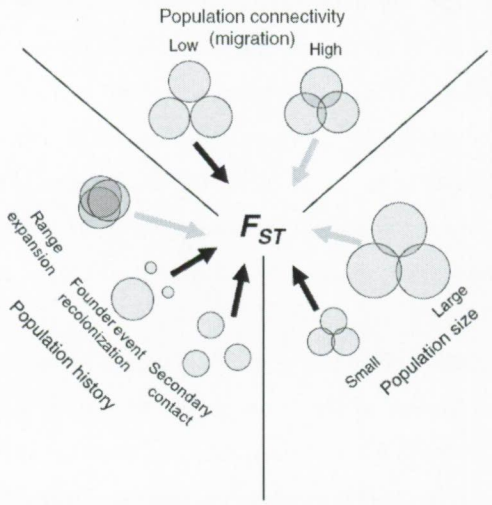

Fig. 3 Factors influencing genetic population differentiation (Hauser & Carvalho 2008). Grey arrows indicate factors reducing strong differentiation; black arrows indicate factors promoting differentiation. Selection is not considered.

- **Challenges in marine species**

Many challenges exist for detecting genetic population structure in marine species. Besides technical aspects such as the difficulty of collecting samples, the time consuming nature of developing neutral genetic markers and the poor fit with traditional theoretical models, marine organisms show biological features reducing genetic differentiation in comparison with terrestrial organisms. Marine organisms, living in environments without any obvious physical barriers, are often characterized by large population sizes, broad geographic distributions, high fecundity, pelagic eggs and larvae and high potential for dispersal. Therefore they are expected to show low levels of genetic differentiation and high gene flow

(Hauser & Carvalho 2008). The magnitude of a subtle genetic structure is often comparable to the noise due to sampling variance. Furthermore, only small amounts of gene flow (1 % in theory) can decrease the level of genetic structure significantly. If one migrant per generation enters a local breeding population, this small amount of gene flow is enough to prevent the accumulation of large genetic differences (Palumbi 2003). Hence the difference between subtle genetic structure and no structure at all is only very small and many marine populations may seem open to gene flow over evolutionary time scales, while they are rather closed over ecological time scales (Palumbi 2004b). Because classical models and metrics like F_{ST} assume constant population sizes, migration rates and equilibrium conditions, these models often do not take into account the heterogeneous and dynamic nature of the sea (Selkoe et al. 2008). More successful is the inclusion of demographic, environmental and oceanographic information to explain differentiation (Francois & Durand 2010; Galindo et al. 2010; Selkoe et al. 2010). The combination of genetic approaches with other tools is called 'seascape genetics' and uses techniques from landscape genetics to test for environmental drivers of spatial genetic structure (Manel et al. 2003; Galindo et al. 2006; Selkoe et al. 2008).

Despite the high mobility and potential dispersal during egg and larval stages, significant population structure in marine fishes does exist (Hauser & Carvalho 2008). Although differentiation in marine fish is much lower than in freshwater species (DeWoody & Avise 2000), there is increasing biological relevance from the correlation with environmental factors (Bekkevold et al. 2005) and the genome-wide characterisation of populations. An overview of population genetic studies on flatfish in the Northeast Atlantic Ocean is presented in Table 1.

Table 1 Literature overview of flatfish population genetic studies in the North-East Atlantic Ocean. Marker type (msat = microsatellite marker, mtDNA = mitochondrial DNA marker, EPIC = Exon-primed Intron-Crossing), sample size, expected heterozygosity (He), genetic differentiation (global F_{ST}), study location and summarized results.

Species	Reference	Marker	sample size	He	Global F_{ST}	Location	Summary
Plaice *Pleuronectes platessa*	Hoarau et al. (2002)	6 msat	24-48	0.74	0.008	11 locations North Sea, Kattegat, Iceland, Biscay, Irish Sea,	* Structure 'continental shelf'-Iceland * No structure North Sea-Irish Sea * Homogeneity within North Sea
Plaice (juveniles) *Pleuronectes platessa*	Watts et al. (2004)	8 msat	6-22	0.39	NA	6 locations Irish Sea, Wadden Sea	* Low genetic diversity * No population structure
Plaice *Pleuronectes platessa*	Hoarau et al. (2004)	6 msat mtDNA (control region)	25-48	0.72-0.76 0.77-0.98	0.035 (msat) 0.102 (mtDNA)	11 locations: NE Atlantic, North Sea, Irish Sea, Baltic Sea	* No IBD * No sex-biased dispersal * Strong differentiation shelf-off shelf with both type of markers * 4 groups only with mtDNA: (1) Baltic, (2) Biscay, (3) Norway, (4) North Sea/Irish Sea
Turbot *Psetta maxima*	Nielsen et al. (2004)	8 msat	26-50	0.56-0.85	0.032 (max pairwise FST)	8 locations NE Atlantic Biscay to Baltic	* Limited/no differentiation within North Sea/Baltic Sea * Structure between North Sea- Baltic Sea
Turbot *Psetta maxima*	Florin & Höglund (2007)	8 msat	30-56	0.64-0.72	0.004	Baltic Sea, Kattegat, Skagerrak	* Low genetic structure within Baltic * No IBD * High temporal variation

Table 1 Continued

Species	Reference	Marker	sample size	He	Global F_{ST}	Location	Summary
Flounder *Platichthys flesus*	Hemmer-Hansen et al. (2007b)	9 msat	18-59	0.23-0.94	0.024	13 locations Baltic + NE Atlantic	* Temporally stable genetic structure * Smaller differences within North Sea
Sole *Solea solea*	Rolland et al. (2007)	3 EPIC markers	30	0.32-0.62	0.043 (Atl-Med) 0.009 (Atl)	8 locations Mediterranean (4) NE Atlantic (4) Bay of Biscay (several locations)	* Genetic homogeneity Denmark to Portugal * Differentiation Atlantic-Mediterranean * No temporal differentiation * East-West differentiation Mediterranean * Adriatic Sea different
Sole *Solea solea*	Guinand et al. (2008)	3 intronic loci	30	NA	0.007	4 locations in Bay of Biscay	* No structure for youngest age classes * Sign.structure for sub-adults * Differences induced by selection?
Flounder *Platichthys flesus*	Florin & Höglund (2008)	7 msat	20-60	0.65-0.95	0.012	20 locations Baltic, Skagerrak, Kattegat	* IBD for pelagic spawners * No IBD for demersal spawners * No clear population structure
Plaice *Pleuronectes platessa*	Was et al. (2010)	8 msat	26-51	0.72-0.81	0.012	Atlantic, North Sea, Irish Sea, Baltic	* Differentiation Iceland-Irish/Baltic * No structure within continental Shelf * Differences between northern and southern part of species range

- **Genetic models of dispersal**

Several spatial genetic models have been defined over the last century to characterize the complex interactions between populations (Hellberg et al. 2002). Among those, the most commonly used classical genetic model is the 'Island model' of Wright (1931), where all populations, equally distant from each other, are linked by equal levels of gene flow, with 'm' migrants each generation. Alternatively, the stepping stone model of Slatkin (1993) is often used, in which only adjacent populations exchange migrants and populations that are closer are linked by large amounts of gene flow. The latter model seems more realistic for many coastal marine species because dispersal is related to geographic distance (Palumbi 2003). Different patterns of geographic genetic structure arise due to the combination of the magnitude of migration rate (m) and effective population size (N_e) (Hellberg et al. 2002) (Fig. 4).

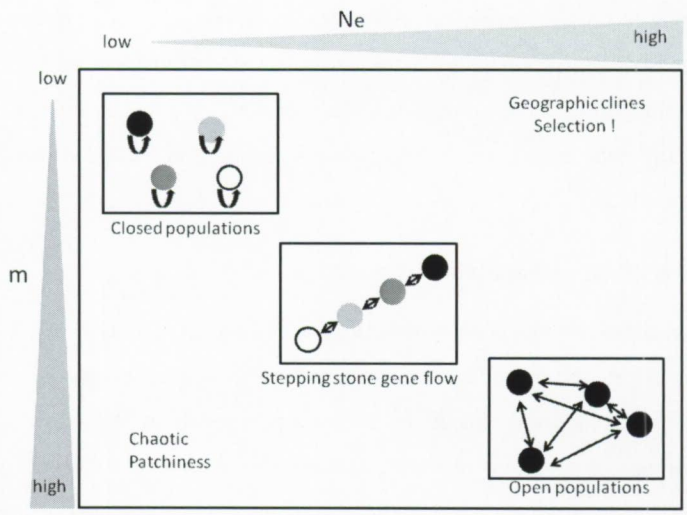

Fig. 4 Patterns of genetic structure arising due to the combination of migration rate and effective population size (Hellberg et al. 2002)

When the effective number of migrants (N_em) is small, populations can be completely closed, with no ecologically meaningful exchange of individuals, but with all recruits coming from within each population. This will result in strong and persistent genetic differentiation between all populations. Population closure may exist in species with strong natal philopatry, even if they have pelagic larval stages. An example of such a marine species

exhibiting strong genetic differences among spawning populations is the mackerel (*Scomber scombrus*) in the eastern Atlantic Ocean (Nesbø et al. 2000). At the other end, when N_em is large, populations can be open, with most recruits coming from other populations. In this case, high genetic similarity is apparent due to ongoing gene flow. When migration only occurs between neighboring populations, a 'stepping stone model to gene flow' exists. This will result in genetic differentiation increasing with geographic distance, also known as an 'isolation-by-distance' (IBD) pattern (a special case of the stepping stone). Many fish and marine invertebrates show such an IBD pattern (Palumbi 2003). In large populations (high N_e), selection may become strong enough to overcome high rates of gene flow, resulting in a geographic cline, a consistent, progressive change in allele frequencies along a geographic axis (Fig. 4) (Hellberg et al. 2002). Finally, many studies have shown that genetic differences between recruits from the same locations are often larger than the genetic differences between adult populations at a larger scale. There are several explanations for this observation. It may result from larvae originating from different sources at different times, from selection at early life stages or from the sweepstakes reproductive success of many species. Due to their high fecundity and high initial mortality, only few adults may produce much of the recruitment during a particular season, resulting in limited diversity of the recruits (Hedgecock 1994).

1.3. Estimating effective population size

The effective population size (N_e) is defined as the size of an ideal population (with an equal sex-ratio, Poisson distributed reproductive success and constant population size) that would lose genetic variation at the same rate as the population under study (Wright 1931). It is an important parameter in conservation genetics because it determines how large populations should be to maintain evolutionary potential ($N_e > 500$) and avoid inbreeding depression ($N_e > 50$) (Frankham 1995a,b). For this reason, changes in genetic diversity should be evaluated in relation to the effective population size rather than the census size N_c (the total number of adult individuals in the population). Although in an ideal population N_e equals N_c, in real populations, N_e is almost always smaller than N_c, causing the ratio N_e/N_c to be less than one. Especially in marine fish, these ratios are extremely low compared to other species (10^{-3} - 10^{-5}) (Hauser et al. 2002; Hutchinson et al. 2003; Hoarau et al. 2005; Poulsen et al. 2006; Chevolot et al. 2008; Larsson et al. 2010) (Fig. 5). The reasons for such small ratio in

marine fish are, amongst others, fluctuating population size over time, unequal sex ratio, social structure and large variance in reproductive success. Especially the latter is identified as an important cause for the low ratio in marine fish (Hedrick 2005). Marine fish are indeed characterized by high fecundity and high mortality early in life (i.e. type III survivorship). The match between the timing of reproduction, the oceanographic conditions and the prey field are crucial (Hjort 1914). Because few parents are actually able to make this match in a given year, offspring will be the result of only a limited number of parents from the parental pool ('sweepstakes reproductive success') (Hedgecock 1994).

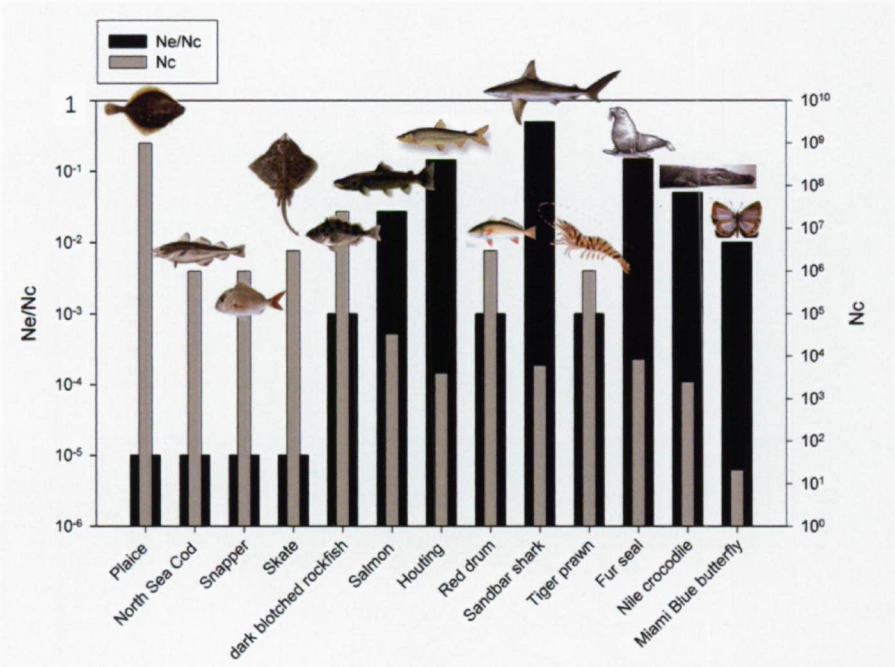

Fig. 5 Ratio of Ne/Nc and Nc for a selection of species (adapted from Portnoy et al. (2010)).

There are two main concepts of N_e, the variance N_e (N_eV) and the inbreeding N_e (N_eI) (Luikart et al. 2010). In the case of a single isolated population of constant size, the two values are equal. Inbreeding N_e deals with the loss of heterozygosity and is influenced by the number of parents while variance N_e considers changes in allele frequency through time and is determined by the number of offspring. N_eV is more sensitive to the detection of population bottlenecks because it decreases more rapidly during a bottleneck.

Several methods are available to estimate N_e; all are based on the expectation that genetic drift increases with decreasing N_e. N_e can be estimated by comparing the variance in allele frequencies at polymorphic loci by repeated sampling across a time frame, separated by a known number of generations. These are the so-called temporal methods (Waples 1989; Wang 2005). Single-sample methods on the other hand, estimate N_e based on a single sample. A general problem is distinguishing between changes in allele frequency due to genetic drift and changes resulting from gene flow (immigration). Most methods assume 'closed' populations without immigration, although some methods exist which take into account Nm (Wang & Whitlock 2003; Waples 2010).

2. Tools for measuring marine connectivity

Different approaches are used for measuring connectivity in marine populations. Ideally, a combination of several tools is used. They include the use of artificial tags, natural tags and biophysical models (Fig. 6).

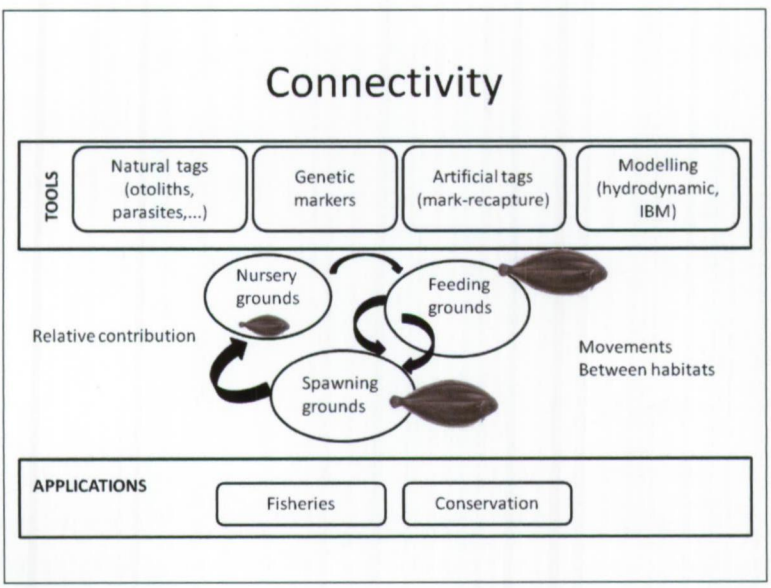

Fig. 6 Conceptual framework of connectivity studies. Several tools are available for measuring connectivity. Connectivity studies assess the movements of individuals between habitats. The results of connectivity studies can be applied in fisheries and conservation.

The use of physical tags such as in mark-recapture studies provide direct information on connectivity but is often limited to adult or large individuals (Palumbi 2004b). Tagging of eggs, larvae or juveniles without introducing handling effects is more challenging. Moreover, recovery rates are often limited. Besides the application of physical tags, artificial tagging can be done by fluorescent compounds (e.g. tetracycline marking) (Jones et al. 1999; Jones et al. 2005) or radioactive isotopes (For an overview see Thorrold et al. (2002)). These methods often require a large proportion of larvae to be tagged and collected and imply the sacrifice of large numbers of individuals. Biophysical models combine physical dynamics and biological traits to investigate connectivity questions (Cowen et al. 2000; Werner et al. 2007; Savina et al. 2010). Advances in individual based modelling (IBM) allow for the incorporation of detailed biological parameters such as larval behavior to quantify dispersal. A review of the possibilities and challenges of biophysical models is given by Werner et al. (2007). They conclude that models should guide the design of field experiments and field observations should improve the model parameters. Natural tags include genetic markers, geochemical signatures and parasites. Parasite faunas tend to vary between populations living in different areas and are often seen as an indicator for demographic independence of stocks (MacKenzie et al. 2008; Sala-Bozano et al. 2009). DNA forms a natural tag present inside every cell of living organisms. By examining the spatial genetic differences between populations, dispersal can be measured. In this thesis we will study connectivity jointly using genetic markers and otolith microchemistry, which is discussed in greater detail below. Both markers have their own temporal resolution: while genetic markers investigate population differences at an evolutionary time scale, otolith microchemical differences are more relevant at an ecological time scale.

2.1. Otoliths as natural tag

Otoliths (ear stones) are paired calcified structures, situated in the inner ear of all teleost fish, used for balance and hearing. There are three pairs of otoliths: saggitae, lapilli and asterisci (Fig. 7). In this work, we will only refer to the sagittae; they are the most frequently used in fisheries science because they are the largest and most easy to locate and handle (Thresher 1999). Otoliths are mainly composed of calcium carbonate ($CaCO_3$) crystals in the aragonite form, surrounded by a protein matrix, called otolin. The elemental composition is dominated by the major elements Ca, O and C, composing the calcium carbonate. These

elements are present in concentrations greater than 10 000 $\mu g.g^{-1}$. Most elements however are present in concentrations > 100 $\mu g.g^{-1}$ (i.e. minor elements) or < 100 $\mu g.g^{-1}$ (i.e. trace elements). Minor elements found in otoliths include Na, Sr, K, S, N, Cl, P while elements such as Cu, Zn, Ba, Cd are detected at trace levels (Campana 1999). The total amounts of inorganic impurities make up less than 1 % of the otolith weight (Panfili et al. 2002). These elemental impurities are either incorporated within the crystal by (i) substitution (e.g. Ca substituted by Sr) or (ii) co-precipitation (e.g. $MgCO_3$); or they can be adsorbed in the interstitial spaces or associated with the protein matrix (Campana 1999, Panfili et al. 2002). The majority of inorganic elements is derived from the water, while a small unknown proportion might be assimilated from the food (Campana 1999).

The detailed physiological regulation of element uptake and otolith growth is beyond the scope of this thesis. It is however important to note that the biomineralization of otoliths differs from that of vertebrate bone, molluscan shell and coral skeletons because otolith formation is an acellular process and the calcification is completely dependent on the composition of the endolymphatic fluid surrounding the otolith and produced by the inner-ear epithelium (Payan et al. 2004). The pathway from the environment to the otolith is a complex route and involves four compartments: (i) the external medium (e.g. seawater, freshwater) where abiotic factors may vary, (ii) the blood plasma of the fish which responds to the external medium but also shows endogenous variation, (iii) the endolymph, the fluid surrounding the otolith, regulating its biomineralization, (iv) the otolith itself, which integrates all these signals (Campana 1999, Thresher 1999, Panfili et al. 2002, Payan et al. 2004).

There are three unique properties of otoliths, which make them very useful in fisheries science. First of all, their growth is continuous. Otoliths are formed at birth and grow each day by the deposition of new layers around the core, by the precipitation of calcium from the surrounding endolymph. The daily and annual rings and specific marks that are formed this way, can be used for age determination, calculations of daily growth or back calculations of length-at-age (Panfili et al. 2002). Second, the concentration of the trace elements broadly correlates with the chemical composition of the seawater (Thresher 1999; Campana & Thorrold 2001). Examples of elements that generally correlate well with the environment are Sr, Zn, Pb, Zn, Mn, Ba and Fe.

Finally, another important property of the otolith which makes it different from other calcified structures such as bones or scales, is that they are metabolically inert; resorption or remineralization is thus negligible. Once material is deposited, it remains unaltered (Campana 1999). Consequently, variability in the trace element composition of otoliths has been used as a natural marker to reconstruct migration histories (Elsdon & Gillanders 2003; Elsdon et al. 2008), discriminate fish stocks (Jonsdottir et al. 2006b; Swan et al. 2006) and trace the natal origin of fish (Warner et al. 2005; Rooker et al. 2008; Fontes et al. 2009).

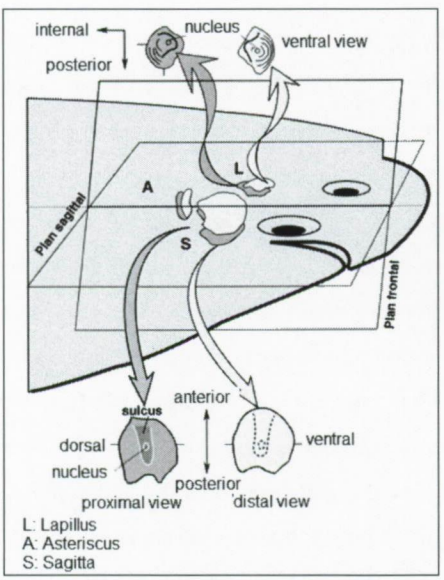

Fig. 7 Position of the three otolith pairs in *Solea solea* (Yves Descatoire, IFREMER 09-97). Left and right sagittae with orientations are presented.

In addition to the elemental composition, otolith shape analysis has become a frequently used tool for species identification and stock identification. Otolith shape is not only species-specific (L'Abée-Lund 1988; Tuset et al. 2006) but also population-specific (Jonsdottir et al. 2006a). Variation in otolith shape is mostly correlated to variation in growth rate among populations (Campana & Casselman 1993). However, differences in otolith shape can also be caused by spatial variation in the feeding conditions, diet, temperature or habitat use.

2.2. Molecular markers in fisheries science

Neutral genetic markers provide estimates of neutral genetic variation, variation with no direct effect on fitness and selection not acting upon the alleles (Holderegger et al. 2006). Neutral markers are highly valuable for estimating the effects of gene flow and genetic drift, for estimating demographic parameters such as effective population size, and for investigating the interaction between demographic processes and their habitat. Adaptive genetic variation on the other hand, refers to the variation in genes with an effect on fitness. Approaches for identifying adaptive divergence in populations of marine fish include (1) the candidate gene approach, (2) the use of genome scans and (3) QTL mapping (Nielsen et al. 2009a). In the first approach, specific genes with a known function that are believed to influence certain traits are screened. In genome scans, large numbers of genetic markers throughout the genome are screened for genetic differentiation and outliers are identified as being potentially under selection. QTL mapping uses a number of markers to build a linkage map of the genetic basis underlying phenotypic traits through a pedigree. Estimates of neutral genetic diversity and differentiation are generally only weakly correlated with adaptive genetic diversity and differentiation (Reed & Frankham 2001; Holderegger et al. 2006). Nevertheless, genetic diversity is important in the long term, to ensure the evolutionary resilience and adaptation potential of the entire species and in the short term, to avoid inbreeding and ensure population survival (Reed & Frankham 2003). In this thesis we used two types of neutral genetic markers: a mitochondrial marker (cytochrome *b*) and one kind of nuclear markers (microsatellite markers).

- **Mitochondrial DNA** (mtDNA) is haploid and only maternally inherited DNA. Because of the lack of recombination, each haplotype (uniparentally inherited) has only one ancestor in the previous generation. Due to its maternally inheritance and haploid character, the effective population size is four times smaller than nuclear DNA and thus it is affected more by random genetic drift. Haplotype frequency differences between populations can be created in relatively short time. Nucleotide polymorphism is considered mainly neutral and thus more influenced by population demography than by selection. For these reasons, mtDNA is a widely used marker in phylogeographic studies (Beebee & Rowe 2008).

- **Microsatellite loci** are tandemly repeated DNA sequences of 2-6 bp long. The number of repeat units varies among alleles. They are co-dominant, highly abundant and randomly distributed throughout the genome. They are considered evolutionary neutral DNA markers. Selection can however affect nearby flanking regions, a process known as genetic hitchhiking. Due to their high levels of polymorphism, relatively small size and rapid protocols, they are widely used in population genetic studies (O'Connell & Wright 1997; Selkoe & Toonen 2006). Challenges associated with microsatellite loci are the development time of suitable markers and the mutation model. Microsatellite loci have mutation rates between 10^{-3} and 10^{-5} per generation and follow most likely a stepwise mutation model (SMM) (Jarne & Lagoda 1996). In contrast to the infinite allele model (IAM), where each mutation leads to new allelic states, the SMM model predicts that mutations occur through the gain or loss of a single repeat unit. Some mutations will therefore result in alleles that already existed in the population (O'Connell & Wright 1997). This creates the possibility of homologous alleles, alleles that are identical in state but not identical by descent (Jarne & Lagoda 1996). Differences between differentiation based on mtDNA and microsatellite DNA can result from sex-biased dispersal or from the difference in genome size (Lukoschek et al. 2008).

3. FISHERIES MANAGEMENT AND CONSERVATION

3.1. The effects of fishing

The direct effects of fishing on a population are obvious. Fishing causes an increase in mortality of both target and non-targeted species. Some unwanted by-catch is discarded at sea, affecting the structure of biological communities (OSPAR 2010). Fishing may also cause a shift in the size and age structure or the sex-ratio of a stock by selectively removing part of the population, which in its turn can have profound effects on the reproductive output (Jennings et al. 2001; Kenchington 2003).

When fishing is selective, it can alter the genetic characteristics within a population. Often these heritable changes in life history traits (e.g. age-at-maturity, length-at-age, number and size of eggs) are irreversible. The removal of large fish is likely to result in slow-growing and early maturing fish because these fish have more chance to survive and pass on their genes

to future generations. Such fisheries-induced changes have been observed in a number of fish such as cod, plaice and sole (Law 2000; Conover et al. 2005; Jørgensen et al. 2007; Mollet et al. 2007). Changes in the genetic diversity of a population under strong fishing pressure can happen within short times. An example is the snapper (*Pagrus auratus*), of which genetic diversity has decreased significantly after tens of years of heavy commercial fishing (Hauser et al. 2002). If fishing pressure is very strong, genetic variation can get lost simply because there are not enough individuals to carry the full range of variability that the population once contained (Hauser et al. 2002; Hoelzel et al. 2006; Lage & Kornfield 2006; McCusker & Bentzen 2010). This will primarily result in the loss of alleles due to genetic drift or in further instance even in the loss of heterozygosity (Allendorf et al. 2008).

Fishing may further cause major changes in marine food webs by altering the mean trophic level. The global trend of the mean trophic level of fisheries landings since the 1950s has been a decline, a concept known as 'fishing down the marine foodweb' (Pauly et al. 1998) (Fig. 8). Fisheries tend to switch from target species at high trophic levels (such as tuna species), to species at lower trophic levels (such as small clupeids) in response to changes in the relative abundances of species. Nonetheless, the use of the mean trophic level of the catches an indicator of the mean trophic level of the marine ecosystem has been questioned because catches are also influenced by changes in economics, fishing technology and targeting patterns (Branch et al. 2010).

Marine fisheries may also alter ecosystem functioning and productivity by altering or destroying habitats (Boehlert 1996). Certain fishing gears, such as the beam trawl, cause physical damage to habitats and the benthic communities (Rabaut et al. 2008). Many coastal marine species have been reduced due to habitat destruction (Briggs et al. online first).
Finally, although species extinctions are not as extensive in marine environments as in terrestrial and freshwater environments, overexploitation has drastically reduced many populations down to 90 % of their pre-exploitation abundance (Briggs et al. 2011). Not only several species of rays, sharks and whales are currently classified as endangered (http://www.iucnredlist.org); many populations of marine teleosts have undergone a collapse (Hutchings 2000, Hutchings & Reynolds 2004).

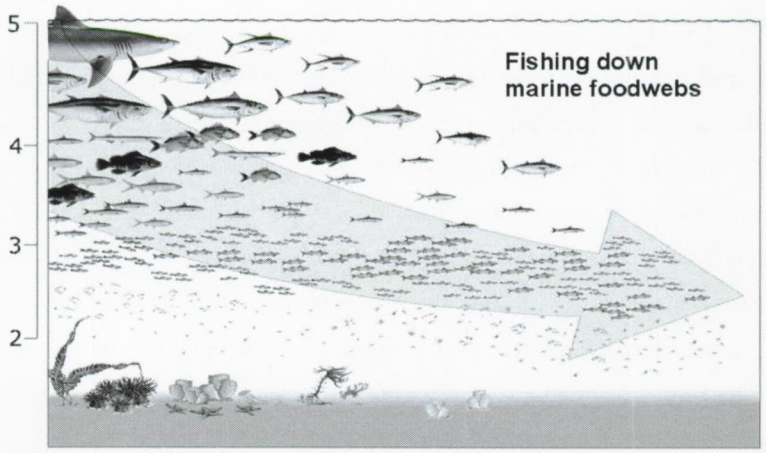

Fig. 8 The mean trophic level of fisheries landings is showing a decreasing trend, a concept known as 'fishing down marine food webs' (Pauly et al. 1998)

3.2. Importance of maintaining genetic diversity

The total genetic variation of a species is partitioned into variation within populations (i.e. differences between individuals) and variation among populations (i.e. differences in the presence and frequency of alleles) (Kenchington 2003). Genetic variation (heterozygosity and allelic diversity) is crucial for both long- and short-term dynamics of populations (Mills 2007). The amount of genetic diversity determines the rate of evolutionary change. Species or populations with very low genetic variation may be unable to adapt to changing conditions (e.g. climate shift, diseases). Maintaining genetic diversity is thus important to maintain full evolutionary potential, as the loss of genetic variation can adversely affect a population by reducing the individual's fitness in the short term. When populations become small and isolated, inbreeding can occur, leading to a loss of heterozygosity and to the accumulation of deleterious alleles. This fitness reduction in the offspring is known as inbreeding depression (Beebee & Rowe 2008). Genetic drift tends to decrease the evolutionary potential of populations through the fixation of deleterious mutations. This is in contrast to natural selection, where only advantageous alleles are transmitted to the next generation because these combinations of alleles are more suited to the environment, leading to local adaptation (the fixation of alleles that are beneficial under local conditions) (see 1.2).

3.3. The value of historical samples

Many fisheries institutes and musea own large collections of scales, bones and otoliths, originally collected for age determination or other purposes. These historical samples may provide a unique source of DNA, which can be used to study long term evolutionary changes. However, historical DNA suffers from degradation due to biological, physical and chemical processes such as nuclease activity and hydrolytic damage (Paabo et al. 2004). Degradation leads to three potential issues to successfully use historical DNA: (1) it is only available in very small amounts compared to recent samples, (2) the length of fragments that can be examined is reduced due to nicked DNA strands (< 250 bp), and (3) DNA damage might lead to genotyping errors or the identification of false mutations (Leonard 2008). For these reasons, it is crucial to develop an optimized method for DNA extraction and storage and to work very carefully to avoid contamination from PCR products or modern DNA. Several guidelines exist for working with historical DNA (Nielsen & Hansen 2008). Essentially, DNA extraction and PCR preparation have to be performed in a dedicated laboratory and negative controls are required at every step.

The analysis of historical DNA has several applications in fisheries science and wildlife conservation, such as the detection of loss of genetic variation or changes in population structure, the evaluation of biological invasions and the estimation of effective population size (Leonard 2008; Nielsen & Hansen 2008). Accurate characterization of historic population size, levels of gene flow and connectivity levels before and after perturbations are fundamental for an appropriate conservation and management. Determining how past populations dealt with environmental changes may help predicting how species and populations today will be impacted by ongoing environmental changes (Leonard 2008).

3.4. Management units and traceability

Management units (MU's) are defined as demographically independent populations whose population dynamics depend mainly on local birth and death rates rather than on immigration (Palsbøll et al. 2007).

In the Northeast Atlantic Ocean, fisheries management is largely based on the European Union Common Fisheries Policy (CFP) of the European Union, which came into force in 1982 and was reformed in 2003 (Reiss et al. 2009). Stocks are mainly managed by setting the total

allowable catch (TAC) and determining some technical measures such as minimum mesh size, effort control and gear restrictions. The International Council for the Exploration of the Sea (ICES) provides scientific advice for setting those measures. The management units defined by ICES are based on geo-economic areas and do not always match within the species-specific biology. For some species, like cod, haddock and whiting, a mismatch between genetic structure and management units is obvious, although there is great uncertainty regarding the consequences of ignoring strong population structure (Reiss et al. 2009).

The European Union is also concerned about illegal, unreported and unregulated (IUU) fishing activities (http://ec.europe.eu/fisheries/iuu). In order to take measures, there is a strong need for certification and traceability tools allowing verification of the legal status of a product. Such tools that not only can identify species but also can trace fish and fish products back to the sampling region are highly valued (Ogden 2008; Martinsohn et al. In Press).

4. Our study system: the Northeast Atlantic Ocean

4.1. Physical environment

The main abiotic factors affecting the distribution of fishes are water temperature, salinity, depth and substrate type. In this section we describe some basic features of the physical environment and oceanography of our study system, the Northeast Atlantic Ocean.

- **The North Sea and English Channel**

The main study area of this thesis is the North Sea, a relatively small and shallow basin with surface area of about 575 300 km², average depths about 30 m and maximal depth down to 200 m (ICES 2005a). The Northern boundary of the North Sea is formed by the Norwegian Deep (down to 700 m depth) and the Southern connection with Atlantic water masses is formed by the English Channel. The North Sea is also connected to the Baltic Sea by the Skagerrak and Kattegat. In winter, the water column is largely vertically mixed, but from spring to autumn, a strong thermocline occurs resulting in stratification in some parts of the North Sea. Salinity ranges from 29 in the southeastern North Sea to more than 35 in the Northwest (ICES 2005a). There is a large freshwater input from major rivers such as the

Rhine, the Scheldt, The Thames, the Wash and Humber, the Weser and Elbe. Along the northeastern English Coast, net average currents are southwards, while along the Belgian and Dutch coast, there is a northward current with an inflow of water from the English Channel (Fig. 9). The coastal waters of Germany and Denmark drift northward. The seabed substrate is variable, but dominated by muddy sands, sands and gravel with some boulder fields in the German Bight and off the Scottish coast.

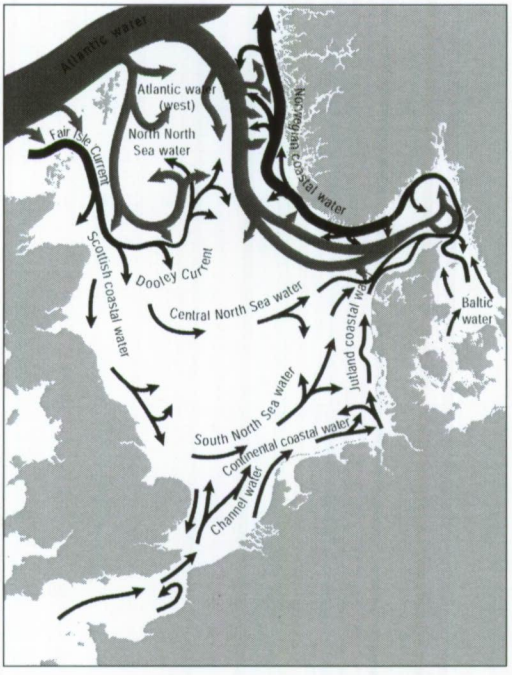

Fig. 9 Schematic overview of the general circulation in the North Sea (OSPAR 2000). The width of the arrows is indicative of the volume transport.

- **Skagerrak and Kattegat**

The Skagerrak is the part of the North Sea, forming the connection with the Baltic Sea. It is a deep and narrow area, with a maximum depth up to 700 m and mean depth of about 210 m (Rodhe 1996). The surface area of the Skagerrak is about 32 300 km^2. The Kattegat is considered part of the Baltic and is much shallower with a mean depth of 23 m. There is a deep current flowing from the Skagerrak into the Kattegat and a surface current transporting brackish Baltic water into the Skagerrak. The surface salinity in the southern Kattegat may decrease down to 15, salinity increases in the Skagerrak and reaches its maximum (30) near the North Sea. The Atlantic current is volumetrically the most important current entering the

Skagerrak, transporting about 1 $km^3.s^{-1}$ of water (Boe et al. 1998). The inflow from rivers and from the Baltic to the North Sea are much smaller than the exchange with the North Sea and the strength of this current flowing into the Skagerrak varies with time (Fig. 9) (Danielssen et al. 1997).

- **Irish and Celtic Sea**

The Celtic Sea represents the transition zone from Atlantic waters to the coastal waters of the Bristol Channel and the Irish Sea. The dynamics of the Irish Sea are dominated by the tides. Surface residual currents are presented on Fig. 10. In the Irish Sea, east of the Isle of Man, a number of gyres are formed and currents are greatly influenced by the wind (Symonds & Rogers 1995). In the Celtic Sea and Bristol Channel, residual currents mainly flow northwards along the coast.

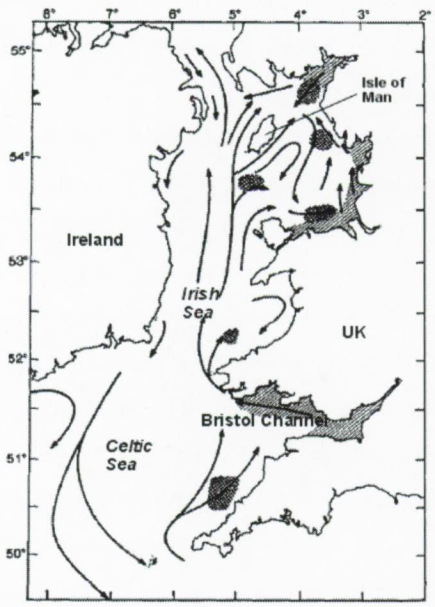

Fig. 10 Direction of the surface residual currents in the Irish Sea, Celtic Sea and Bristol Channel with inshore nursery grounds and main spawning grounds indicated as shaded areas (From: Symonds & Rogers (1995))

4.2. Environmental differences in water chemistry

The application of otolith microchemistry for stock discrimination implies that the environments in which the fish live are chemically different. Even so, water chemistry data are difficult to link to concentrations measured in otoliths due to the many physiological

processes during element uptake (Campana 1999, Panfili et al. 2002). Several studies have analyzed variation in the dissolved metals in the study area (Baeyens et al. 1987, Scholten et al. 1998, Baeyens et al. 2005). The most frequently monitored heavy metals are cadmium (Cd), Mercury (Hg) and lead (Pb) because of their toxicity (OSPAR 2010). An actual comparison between reported values was difficult because data were obtained from different years, seasons or limited geographical areas and produced by different methodologies.

Changes in the dissolved trace metal concentrations in the water may result from changes in river inputs, atmospheric inputs, differences in sediment type, hydrodynamics and/or differences in bio-geochemical processes (Scholten et al. 1998). Heavy metals may also reach the North Sea via human activities such as dredging, dumping, shipping and petroleum industry (OSPAR 2000). In general, trace metal concentrations in the water and in sediment are highest along the coast and decrease towards the open sea, suggesting that rivers are the major sources of trace metals (OSPAR 2000).

Regional differences in trace element concentrations (Cd, Cu, Zn and Pb) in sea water were found in the North Sea during the period 1980-1989 (Scholten et al. 1998). The German Bight and Dutch coastal area contained high amounts of all four metals. The Skagerrak region was mainly enriched in Pb and Cu, while the east coast of the UK showed high concentrations of Cd. Concentrations of dissolved Mn in the Wadden Sea were distinctly higher than in the German Bight in the period 2002-2003 (Dellwig et al. 2007). Manganese enrichments at the tidal flats in the Wadden Sea were especially pronounced during summer due to increased microbial activity in the sediment. Major Mn sources for the Wadden Sea are the pore water draining out of the tidal flat sediments during ebb tide and the freshwater contribution (Dellwig et al. 2007). The river Thames has a long history of pollution and exhibits high concentrations of Ag, Cd, Pb, Hg, Cr in the sediment (Attrill & Thomes 1995). A study by Power et al. (1999) analyzed trends in the concentrations of Cd, Cu, Hg, Ni, Pb and Zn in the surface water between 1980 and 1997 and observed reductions for all elements except Cd and Pb. The Belgian coastal zone is strongly influenced by the eutrophic rivers Scheldt, Rhine and Meuse. Dissolved and particulate metal concentrations (Cd, Cu, Pb, Zn) were measured in the period 1978-1995 in the Scheldt (Baeyens 1998). Especially Cd concentrations were very high in the Scheldt (Baeyens 1998). Based on the trace metals Ni, Cd, Cu, Zn and Fe in the water and Mn in suspended matter, the Rhine

estuary, the Dutch coastal waters, the centre of the Southern Bight and the English coastal waters could be distinguished (Nolting 1986). Recent assessments show significant reductions in the input of Cd, Hg and Pb over the period 1990-2002 in several regions of the North Atlantic (OSPAR 2005, OSPAR 2010). The reported chemical differences in sea water are promising for the application of otolith microchemistry for stock discrimination within the North Sea.

4.3. The North Sea fishery: from past to present

The first references of trawl fishing date back to the 14th century, when the use of a new fishing gear (beam trawl) was prohibited because of its detrimental effects on the seabed and its organisms (Engelhard 2008). Nevertheless, the development of the trawl continued constantly. In the 19th century, the North Sea was mainly exploited by sailing vessels, trawling a small wooden beam and fishing for flatfish and roundfish. During the 1870s, trawling by sail reached a peak. After 1880 the development of steam power became more important allowing trawlers to stay longer time at sea. Trawling by sail was gradually replaced by steam-powered vessels which dominated the first half of the 20th century. After World War I, tickler chains came into use, increasing the catch efficiency by 25 % (Rijnsdorp et al. 2008). During both world wars, there was a significant reduction in trawling effort and landings because of restrictions on vessel movements. As a result, catch rates immediately after these years returned to very high levels. Because of various technical innovations (e.g. diesel engines replacing coal and oil engines) the steam trawl fleet was replaced by a motor trawl fleet from the 1950s onward. The active gear used with these motor trawlers was the otter trawl. Developments such as onboard freezing facilities made it possible to stay at sea for a longer time and exploit more offshore areas (Engelhard 2008). In the early 1960s, the modern twin-beam trawl was introduced, consisting of two large beam trawls up to 12 m on each side of the ship and equipped with tickler chains (Fig.11). The method was especially adopted by the Belgian, Dutch and German fishery and very effective in catching flatfish, in particular sole (Rijnsdorp et al. 2008). Sole played an important role in the development of this fishery and the very strong year classes of 1958, 1963 and 1969 resulted in high catches, encouraging the construction of even more vessels with a trend towards increasing engine power.

Whereas the number of otter trawlers in the Belgian fleet decreased gradually in favour of beam trawlers, beam trawling was introduced to the English North Sea fisheries only in the 1980s (Engelhard 2008).

Fig. 11. Evolution from sailing vessel (upper left) to steam vessels (upper right), to diesel powered vessels equipped with large beam trawls (lower panels). First three pictures, © VLIZ 2010

An overview on the historical fisheries landings in Belgium is presented in Lescrauwaet et al. (2010). From these data it becomes clear that sole has been an important species for the Belgian fisheries over the period 1929-1999, in terms of value of the landings.

In recent years, increasingly sustainable and selective fishing gears are encouraged to reduce the bycatch and destructive environmental effects of beam trawling (Depestele et al. 2009). Recent years exploitation levels have decreased with some indication of recovery, in particular in plaice (*Pleuronectes platessa*) (www.ices.dk).

Sole are mainly caught in a mixed beam trawl fishery with plaice and other flatfish using 80 mm mesh in the southern North Sea. The minimum mesh size in the mixed beam trawl fishery in the southern North Sea means that large numbers of undersized plaice and cod are discarded. The minimum landing size is 24 cm.

The spawning stock biomass has been fluctuating around the precautionary reference point of 35000 tonnes (Fig.12). Recruitment is highly variable and dependent on strong year classes (e.g. 1963, 1987, 1997, 2001). The total allowable catch was 14000 tonnes in 2009. Fishing mortality has been above the precautionary reference limit of 0.4 during the period 1967 – 2007 and is showing a decreasing trend during the last few years (Fig. 12) (www.ices.dk).

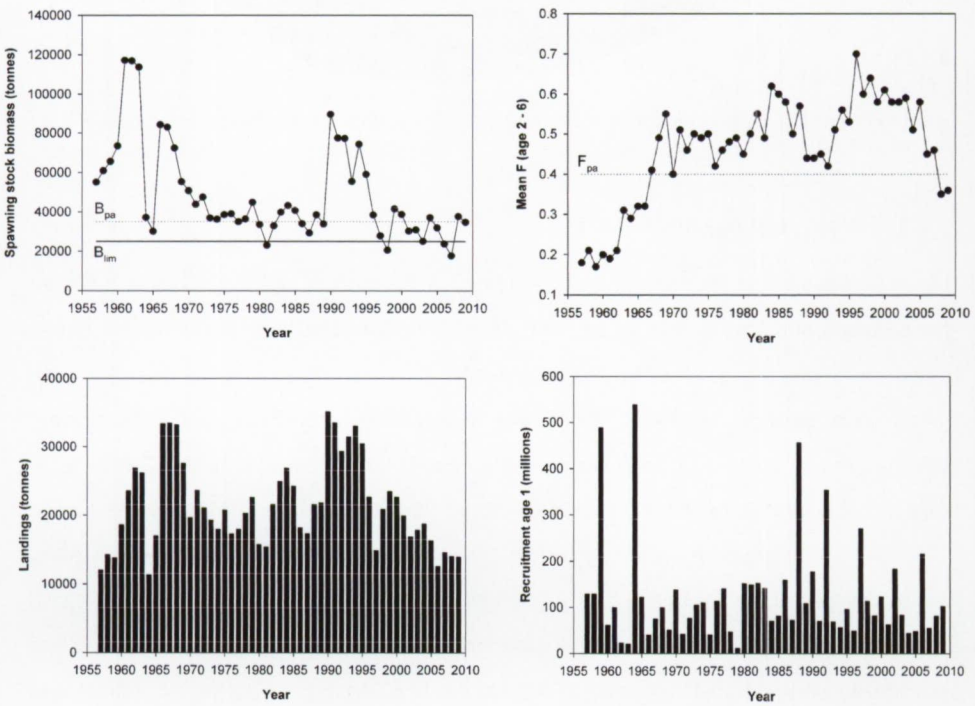

Fig. 12 Sole in ICES subarea IV. Summary of the stock assessments. Evolution of spawning stock biomass, fishing mortality, landings and recruitments (data from ICES 2010). Reference points: B_{pa} = precautionary approach spawning stock biomass, B_{lim}= limit spawning stock biomass, F_{pa}= precautionary approach fishing mortality

5. SOLE AS A MODEL SPECIES

Class Actinopterygii
Ordo Pleuronectiformes
Family Soleidae
Solea solea Linnaeus, 1758 (Fig. 13)

Fig. 13 Picture of sole (*Solea solea);* note the dark patch on the pectoral fin, the subterminal mouth and the continuous dorsal fin up to the tail.

5.1. Biology, ecology and fishery

Sole mainly live in the southern and eastern North Sea, south of an imaginary line between Flamborough and the Danish coast. This line corresponds to the position of a steep temperature cline; it divides the North Sea into a cold stratified northern section with bottom temperatures of about 7°C and a warm mixed southern part with bottom temperatures of up to 17°C in summer and autumn (Rijnsdorp et al. 1992). The detailed biogeographical range of sole extends in the south from the northwest African coast (up to 12°N), the Mediterranean (including Sea of Marmara, Bosporus and south western Black Sea) to the Gulf of Biscay. In the North it ranges from the Skagerrak and Kattegat, the Irish Sea to the southern North Sea. Sole are sometimes caught in low numbers off Scotland and occasionally along the southern coast of Norway (up to 62°N) (Fig. 14). Older individuals tend to occur in deeper waters than the juveniles, but they remain largely restricted to waters less than 50 m deep (Rijnsdorp et al. 1992). Sole show sexual dimorphism, with females attaining a larger size than males at a given age. The maximum recorded length is 70 cm and maximum recorded age 40 years, although recent catches are dominated by 2 and 4 year old fish due to the high exploitation level (www.ices.dk).

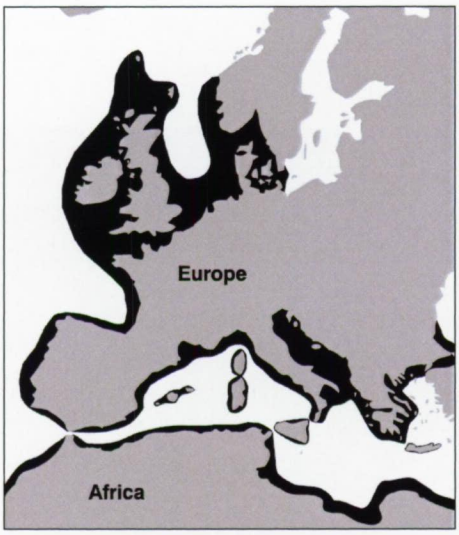

Fig. 14 Distribution range of *Solea solea* (after Horwood (2001))

As most flatfishes, sole is a batch spawner, releasing several successive batches of eggs (Rijnsdorp & Witthames 2004). There are five main spawning hotspots in the North Sea: (1) the inner German Bight; (2) in the eastern Channel; (3) off the Belgian coast; (4) in the Thames estuary and (5) on the Norfolk Banks (De Clerck & Van de Velde 1973; Borremans 1985; Rijnsdorp et al. 1992; Wegner et al. 2003) (Fig. 15). Spawning grounds are limited by the 30 m depth contour. The peak of spawning is from March in the Bay of Biscay to late May in the southeastern North Sea. Spawning is triggered by sea water temperature in the North Sea and the peak in egg production is advanced in warm springs (Rijnsdorp et al. 1992). In the Bay of Biscay spawning occurs further offshore than in the North (Koutsikopoulos & Lacroix 1992).

Development time of the pelagic eggs is temperature dependent. At a temperature of 10°C-15°C eggs hatch after seven to eight days. The pelagic larvae feed on copepod nauplii and dinoflagellates (Russell 1976; Fonds 1979). Larvae settle during metamorphosis at a length of 7-10 mm about three weeks after hatching (Fonds, 1979). The duration of the planktonic phase is determined by ambient water temperature and food availability but lasts normally four weeks (Rijnsdorp et al. 1992). Ichthyoplankton surveys carried out in the eastern English Channel and the Southern Bight of the North Sea suggest that sole larvae are retained in

coastal areas throughout their development, despite strong hydrodynamics (Grioche et al. 2001). It is probable that in some areas they use vertical migration to resist water advection (Champalbert & Koutsikopoulos 1995; Grioche et al. 2001).

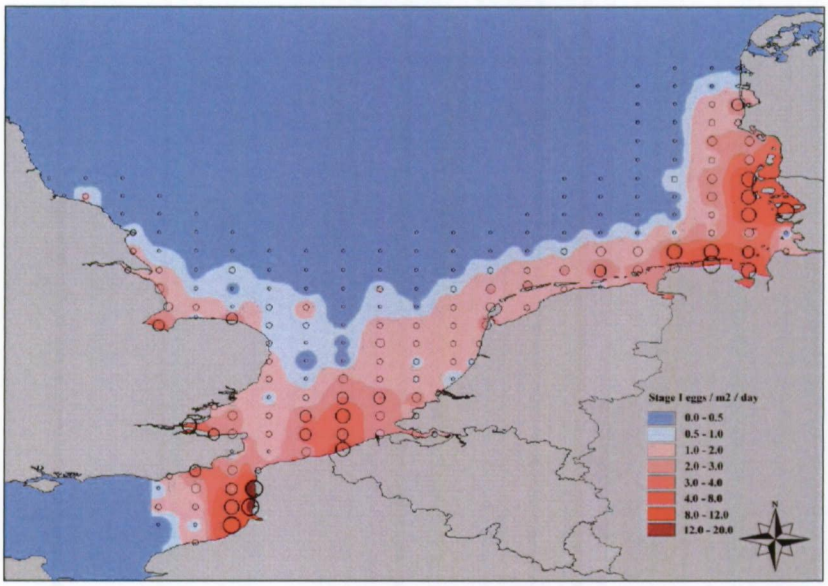

Fig. 15 Indication of the spawning areas of *Solea solea* based on the occurrence of eggs. Coulours indicate egg density (number of eggs.m^{-2}.day^{-1}) (From: ICES 2005a 'Fishmap Sole').

Once metamorphosis has been completed, sole join the 0-group (Horwood 2001). Nursery grounds are distributed in shallow sandy or muddy coastal areas with reduced salinity (sediment particle size 4 to 500 µm; depth to 20 m but mostly less than 10 m) (Rijnsdorp et al. 1992, Horwood 2001). The Wadden Sea is considered one of the most important nursery grounds. Between age 3 and 4, sole move offshore and recruit into the local spawning stock. When water temperature decreases during winter, sole migrates into deeper waters. Adult sole occur in the summer in waters to 40 m and mostly in places with fine sediment. Natural mortality can be extremely high when water temperatures drop below 4°C for an extended period (Woodhead et al. 1964 a,b). Sole are nocturnal feeders on worms (particularly *Arenicola marina*, *Lanice* spp. and *Nereis* spp.), mollusks and small crustaceans (Braber & de Groot 1973, Lagardère 1987). During the day, they are burrowed into sandy and muddy bottoms.

Sole populations can be split into biologically relevant groups based on differences in life-history traits with latitude. Fecundity increases with latitude, from 200 000 eggs around Portugal to 450 000 eggs in the German Bight (Witthames et al. 1995). Additionally, there are differences in fecundity between the Western (Flamborough) and the Eastern (German Bight) part of the North Sea. The size of the eggs, length, weight and age at maturity vary according to population location (Rijnsdorp & Vingerhoed 1994) (Table 2). These data indicate the probability of genetically isolated subpopulations. Separate populations seem to occur in the Irish Sea and Bristol channel (Rijnsdorp & Witthames 2004).

Table 2 Life-history traits of *Solea solea* stocks according to latitude and longitude (From Rijnsdorp & Witthames (2004). Fecundity in 1000 eggs for a female of 35 cm. Peak spawning is counted in days since 1st of January. Length Lm (cm) and weight Wm (g) at maturity and egg size (mm).

Location	Lat (°N)	Long (°E)	Fecundity	Peak spawning	Lm	Wm	Egg size
Skagerrak/Kattegat	58	10	-	-	25	154	-
German Bight	54	5	440	148	-	-	1.132
Flamborough	54.3	0	258	151	-	-	1.257
Irish Sea	53.3	-4	342	-	32	323	-
Texel	53	3	432	131	33	355	1.226
Eastern English Channel	50.3	0.3	325	111	28	-	1.264
Western English Channel	49.3	-4.3	240	91	28	-	1.369
Gulf of Biscay	47	-3	221	60	35	423	-

Tagging studies support the hypothesis that, once recruited to a spawning ground, sole continue to spawn on that ground year after year ("homing"). It is not known whether recruits return to the grounds where they were born ("natal homing") (Horwood 2001). Tagging experiments of juveniles in a number of nursery areas in the English Channel and North Sea have shown that each spawning population is mainly supplied by recruits from the nearby nursery ground. Recaptures in the spawning season show that sole tagged in the North Sea do not substantially recruit to the English coast and *vice versa* (Rijnsdorp et al. 1992). Sole undertake relatively short movements away from their coastal nursery and

spawning grounds with average distances of 75 km for mature fish around the spawning period and about 150 km during the rest of the year. The direction of the movements was mainly northwards in the North Sea while it was mainly westwards in the Eastern English Channel, mirroring the direction of the currents (Burt & Millner 2008).

5.2. Population genetic studies

Despite its commercial importance, population genetic studies on common sole are relatively scarce. Most of the previous studies suffer from low sample size or low statistical power due to limited number of markers. In general, Atlantic populations are considered as one panmictic unit (Rolland et al. 2007). In the North Atlantic Ocean, a weak isolation-by-distance pattern has been observed by some authors with allozyme markers (Kotoulas et al. 1995; Cabral et al. 2003). The geographical unit of population structure (corresponding to a panmictic unit) has been roughly estimated at 100 km by Kotoulas et al. (1995). Both allozyme and RAPD data indicate similarity between Bay of Biscay and German Bight populations and between Irish Sea and English coast (East Anglia) (Exadactylos et al. 1998). Furthermore, there is a separation between the coast of the British Isles and continental Europe (German Bight/Biscay). It has been suggested that the depth of the Dover Strait (50-100 m) might prevent adult sole from crossing the Channel (Exadactylos et al. 2003). In the Bay of Biscay, temporal genetic stability has been observed between the 0-group and 1-group but significant differences existed among subadults, suggesting that differentiation might arise over time (Exadactylos et al. 1998; Rolland et al. 2007; Guinand et al. 2008). Differential selection acting at the nursery grounds could create the observed differences among subadults (Guinand et al. 2008).

The genetic differentiation between sole populations from the Atlantic and from the Mediterranean is unambiguous and has been documented with allozyme markers, EPIC markers and cytochrome *b* (Cabral et al. 2003; Rolland et al. 2007; Maes & Volckaert pers.comm.). Within the Mediterranean region, differentiation has been observed between eastern and western Mediterranean and also between Adriatic Sea and Mediterranean using allozymes and EPIC markers (Kotoulas et al. 1995; Rolland et al. 2007). This fits with the general observation for many marine taxa inhabiting the Mediterranean Sea (Patarnello et al. 2007).

A high degree of spatial differentiation was also found using the control region of mtDNA between subpopulations in the central Mediterranean sea and those from the northern basins (Tyrrhenian Sea, Adriatic Sea, Ligurian Sea) (Guarniero et al. 2002). *Solea* is thought to have originated in the Atlantic Ocean and to have entered the Mediterranean Sea during the early Pliocene at the end of the Messinian salinity crisis (Borsa et al. 1997) when contact was reestablished between the Atlantic Ocean and Mediterranean Sea. The pattern is still recognizable in the genetic variation in an East – West direction (Kotoulas et al. 1995; Garoia et al. 2007).

5.3. Otolith microchemistry and stable isotope studies

Juvenile sole occur in high numbers in estuarine and coastal nurseries. These areas receive anthropogenic input from rivers, resulting in environmental differences in water chemistry. Because this increases the likelihood of detecting spatial differences in otolith microchemistry, most studies on sole microchemistry have been done in the context of estuarine immigration and connectivity among nursery grounds. For instance, sole juveniles from the main estuaries of the French Atlantic coast could be discriminated by the elemental fingerprints of their otoliths (de Pontual et al. 2000). Additionally, de Pontual et al. (2003) studied the influence of ontogenic stages on the Sr/Ca ratio in sole and observed that metamorphosis has an effect on microchemistry signals. Distinct otolith fingerprints were also obtained for juvenile sole caught at several estuarine nursery grounds along the Portuguese Coast (Vasconcelos 2007) and in a later study these fingerprints were used as a baseline for determining the nursery origin in adult sole (Vasconcelos et al. 2009). The authors concluded that adult sole along the Portuguese coast seemed to have mixed nursery origins (Vasconcelos et al. 2009). Juvenile sole from the Thames estuary also appeared to be characterized by low mobility during the first year. This was shown using otolith microchemistry based on 14 elements, enabling the identification of estuarine versus coastal nursery origin of fish (Leakey et al. 2009). Similar results were obtained with stable isotope analyses where there was a consistent relationship found between isotopic signature ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) of juvenile sole and their invertebrate prey (Leakey et al. 2008). Distinct isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) pointed towards high site fidelity in 0-group sole in two Portuguese estuaries (Vinagre et al. 2008b).

5.4. Modelling studies

A particle-tracking model coupled to a 3D hydrodynamic model was developed for sole by Savina et al. (2010). Four types of vertical movement of larvae (one passive behavior and three types of active behavior) were tested to assess larval retention and connectivity in the southern North Sea (release positions, Fig. 16).

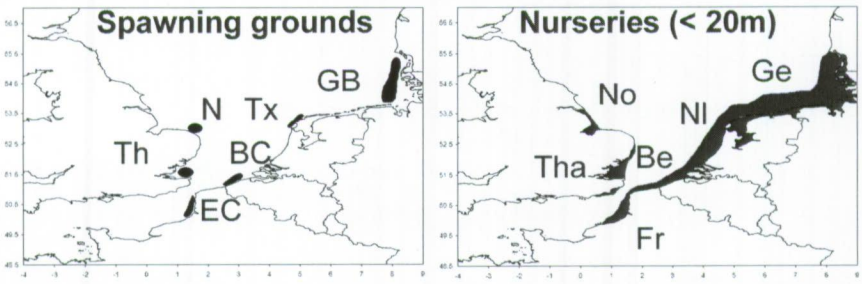

Fig. 16 Left: 6 main spawning grounds (release positions) in the eastern English Channel and the North Sea (Eastern English Channel (EC), Belgian Coastal (BC), Texel (Tx), German Bight (GB), Norfolk (N) and Thames (Th)). Right: nurseries defined as the coastal areas where depth < 20m and delimited by national boundaries (French nursery (Fr), Belgian nursery (Be), Netherlands nursery (Nl), German nursery (Ge), Norfolk nursery (No) and Thames nursery (Tha)) (Savina et al. 2010).

Results suggest that spatial variation in the retention of larvae above nursery areas is influenced by the hydrodynamic conditions. Also the effect of larval behavior on the retention seems to differ in space. The variability of retention due to the hydrodynamic variability is dominant. Simulations with passive larvae show that the transport from the English Channel, the Belgian Coast and Texel is directed north-eastward along the Belgian and Dutch coast. The transport from the German Bight is oriented north westward along the coasts of Germany and Denmark. Transport from the Thames is oriented eastward, offshore and from the Norfolk Banks north-eastward away from the coast (Fig. 17).

Vertical migration behavior that includes tidally synchronized movements of larvae in the water column enhances the northeastward transport of larvae from the English Channel, the Belgian Coast and the Thames. Results suggest a moderate exchange of larvae and a relatively high amount of retention between the various spawning areas (Fig. 18).

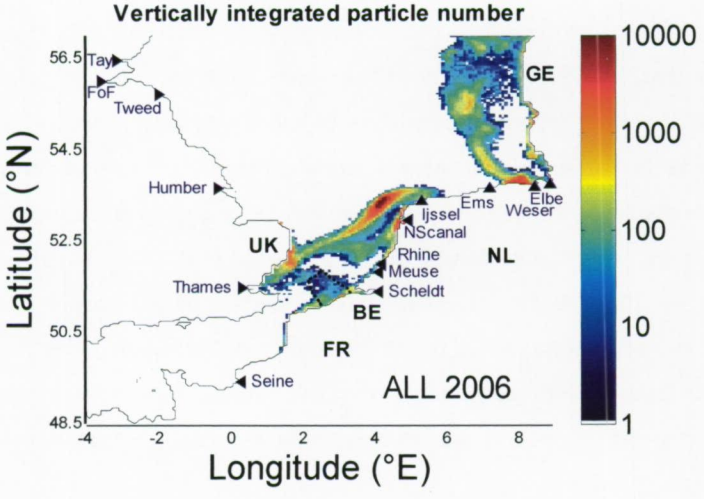

Fig. 17. Final distribution of sole larvae at the end of the pelagic phase (number individuals/grid cell) for eggs spawned in the six spawning grounds (Eastern English Channel, Belgian Coast, Texel, German Bight, Norfolk and Thames) in 2006. (updated from Savina et al. 2010).

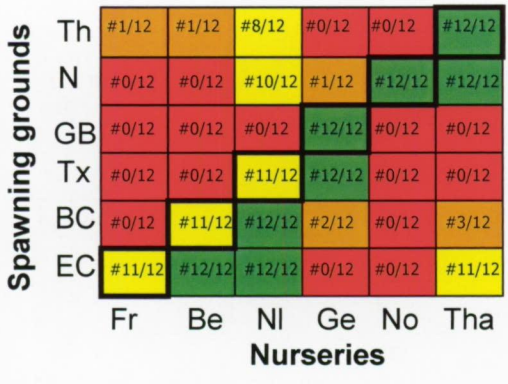

Fig. 18. Frequency of connections between nurseries and spawning grounds. The number represents the number of years where connections have been simulated (over 12 years). Green: always, yellow: frequent, orange: sometimes and red: never. Spawning grounds: Thames (Th), Norfolk (N), German Bight (GB), Texel (Tx), Belgian Coast (BC), Eastern English Channel (EC). Nurseries: French coast (Fr), Belgian Coast (Be), Dutch coast (Nl), German Bight (Ge), Norfolk coast (No), Thames (Tha). (updated from Savina et al. 2010).

In addition to hydrodynamic models, GIS habitat models provide a tool for modeling species distribution in unknown areas with known environmental factors (Koubbi et al. 2006). For *Solea solea*, these studies have identified the depth of the water column and the seabed sediment as the most important descriptors of juvenile nursery habitat in the southern North Sea and the eastern English Channel (Eastwood et al. 2003; Reed & Frankham 2003). Juvenile sole are concentrated in shallow muddy areas, including estuaries, because these habitats have high invertebrate prey productivity (Le Pape et al. 2003a; Le Pape et al. 2003b; Le Pape et al. 2007). Depth, sediment, salinity and temperature were also the most important factors that were related to the egg and larval distribution during spawning. Sole in the English Channel preferred to spawn in shallow waters of lower salinity, above a certain minimum temperature (7-10°C) and in areas with sediment containing < 30 % gravel (Eastwood et al. 2001).

6. Objectives and outline of the thesis

The main aim of this thesis was to contribute to the knowledge of the spatial and temporal connectivity of sole (*Solea solea* L.) in the Northeastern Atlantic Ocean. The thesis is subdivided into two main parts. In the first part, the complex contemporary population structure of sole is investigated using a multidisciplinary approach. A combination of genetic markers and otolith microchemistry and shape was used, offering a complementary and powerful strategy to study migration patterns. In the second part we studied the genetic demographic stability of North Sea sole using a temporal time series of historical DNA.

Part I

In **Chapter 1** the contemporary population genetic structure of sole was studied in the North-East Atlantic Ocean using a set of microsatellite markers and a mitochondrial marker (cytochrome *b*). The following research questions were investigated: Are the different spawning aggregations genetically differentiated? Are juvenile sole from different nursery grounds genetically different and are they similar to the nearby adult spawning group? In chapter 1 we also analyzed the temporal stability of the genetic pattern observed. Results are interpreted on an evolutionary time-scale to try to define long-term management units.

At the ecological time-scale, otolith markers were applied to investigate migration patterns in *Solea solea*. **Chapter 2, 3 and 4** present the results of the otolith microchemistry work and the otolith shape analyses that we performed. Initially, the elemental fingerprints of juvenile sole were characterized and spatial variation in chemical composition was analyzed (**Chapter 2**). Next, adult sole otoliths were analyzed using otolith microchemistry and shape analysis to assess their potential for tracing fish back to their capture site and to study the existence of ecological units in sole (**Chapter 3**). In **Chapter 4**, we analyzed the chemical composition of the juvenile section of adult otoliths and compared it to the juvenile baselines obtained in chapter 2 to evaluate the nursery origin of adult sole. **Chapter 5** presents a multidisciplinary approach to study population structure and traceability in sole. The chapter combines and integrates results from genetic markers with those from otolith markers, some of them collected on the same fish.

Part II describes the results of the analysis of historical DNA from an otolith collection dating back to 1957. Before long-term temporal changes in genetic diversity can be studied, it is important to develop a method suitable for the DNA extraction from those valuable historical otoliths. Therefore, we first optimized a protocol for DNA isolation and evaluated the harmful effects of some extraction methods on the successive age and growth analyses (**Chapter 6**). Following the optimization of the DNA extraction, we analyzed the long time series (**Chapter 7**). Samples were genotyped with microsatellite markers and genetic variation and differentiation was examined among sampling years and among cohorts. In this chapter we investigated if neutral genetic diversity has changed over time, in relation to the increase in fishing pressure. Another aim was to provide an estimate of effective population size of sole.

Finally, the results from all chapters are combined in an **integrated general discussion** where we try to describe the population model that fits the best for sole. The implications of these results for fisheries management and conservation are briefly addressed. To conclude, perspectives for future research are proposed.

Connectivity in *Solea solea*

Part I

Chapter 1

Multi-marker estimate of genetic connectivity of sole (*Solea solea*) in the North-East Atlantic Ocean

Cuveliers E.L., Maes G.E., Hellemans B., Verherstraeten S.L.N.A., Larmuseau M.H.D. & Volckaert F.A.M.

ABSTRACT

Gaining a better knowledge on genetic connectivity of marine populations is important in fisheries management and conservation, to validate the patterns observed with more direct methods such as tagging studies or larval dispersal modelling studies. The use of multiple genetic markers allows to test for the influence of historical and contemporary divergence between populations and to discriminate between sex-biased dispersal and effective population size effects. Here, we examine the population genetic structure of sole (*Solea solea* L.) in the North-East Atlantic Ocean using ten nuclear microsatellite loci and a 590 bp fragment of the mitochondrial cytochrome *b* gene to infer the genetic connectivity between the main populations and the concordance with current management units. Both markers point to significant spatial differentiation between the most Northern samples (the Kattegat, Skagerrak, and Belt Sea) and the Southern North Sea/Bay of Biscay. The Irish and Celtic Sea showed also some genetic differences with the North Sea and with the Kattegat/Skagerrak. Within the North Sea, genetic differences were more subtle and not temporally stable. Despite low levels of differentiation, a pattern of isolation by distance was observed with nuclear markers, pointing to a dispersal pattern concordant with the prevailing currents in that region. Our results suggest that three more or less isolated groups exist in the NE Atlantic, while biological populations within the Southern North Sea are likely connected by sufficient gene flow or may have diverged too recently for significant genetic differences to become evident.

INTRODUCTION

Population connectivity plays a fundamental role in the dynamics of marine ecosystems (Jones et al. 2007). Knowledge on the dynamics of local populations is a prerequisite for an effective and sustainable fisheries management (Hedgecock et al. 2007). The delineation of appropriate management units requires information from population genetic, ecological and demographic data (Palsbøll et al. 2007). Additionally, such information is crucial for maintaining a safe level of intra-specific diversity (Reiss et al. 2009) and for the design of marine reserves (Fogarty & Botsford 2007; Jones et al. 2007).

Marine fish with large population sizes, broad geographic distributions, high fecundity and high dispersal potential are expected to show low genetic differentiation and high gene flow (DeWoody & Avise 2000). Although a number of population genetic studies found evidence for structure even in species with high dispersal potential (Knutsen et al. 2003; Nielsen et al. 2004; Pampoulie et al. 2004; Jørgensen et al. 2005), measuring genetic connectivity in marine environments without any obvious physical barriers remains a challenge. Besides high levels of gene flow, other factors, such as the recent recolonization events and high effective population size of marine fish could explain the lack of detectable genetic differentiation at neutral loci (Buonaccorsi et al. 2001). Many population genetic studies have used microsatellite markers for the detection of subtle population structure, because of their high levels of polymorphism (Selkoe & Toonen 2006). However, due to its haploid character and maternal inheritance, the effective population size of mitochondrial DNA is four times smaller than that of nuclear microsatellite loci, making mtDNA more susceptible for the effects of genetic drift. Estimates of population differentiation are indeed often higher for mitochondrial markers (Hoarau et al. 2004; Lukoschek et al. 2008; Larmuseau et al. 2010).

The target species of this study, the sole (*Solea solea* L.), has a wide geographical distribution range from the southern coast of Norway, the Kattegat and the Skagerrak to the Northwest African coast, also including the Mediterranean Sea. Some biological characteristics might prevent strong population structure in sole. North Atlantic sole spawn a high number of pelagic eggs, hatching after 7-8 days. The pelagic larvae settle about three weeks after hatching (Fonds 1979) in nursery areas, distributed in shallow sandy or muddy coastal areas

with lower salinity (Rijnsdorp et al. 1992; Horwood 2001). Other characteristics however point to the possible existence of subpopulations. First, differences in life history traits (e.g. number and size of the eggs) exist among subpopulations of sole across a latitudinal gradient (Rijnsdorp & Vingerhoed 1994; Witthames et al. 1995). It remains unclear whether these differences exist due to phenotypic plasticity or local adaptation. Second, several distinct spawning aggregations have been identified based on plankton surveys. In the North Sea these spawning hotspots are situated in the inner German Bight, in the Eastern English Channel, off the Belgian coast, in the Thames estuary and on the Norfolk Banks (De Clerck & Van de Velde 1973; Borremans 1985; Rijnsdorp et al. 1992; Wegner et al. 2003). The peak of spawning is from March in the Bay of Biscay until May-June in the Southeastern North Sea and the Irish Sea (Symonds & Rogers 1995). Third, a study modelling the transport of sole larvae in the Southern North Sea indicated that in general the exchange of larvae among spawning populations is much lower than the amount of larvae retained (Savina et al. 2010). Finally, tagging studies showed limited juvenile movement away from their spawning and nursery grounds (Burt & Millner 2008), which is also confirmed by a microchemistry study on juvenile sole from the same study area (Chapter 2). The southern Bight of the North Sea is currently considered one management unit (ICES area IV) (Reiss et al. 2009).

Despite the commercial importance of sole, population genetic studies in the North-East Atlantic Ocean are relatively scarce, compared to studies in the Mediterranean Sea and the Bay of Biscay (Guarniero et al. 2002; Cabral et al. 2003; Garoia et al. 2007; Guinand et al. 2008). In the NE Atlantic, an isolation-by-distance pattern (IBD) was found from the English Channel to the Tunesian Coast with allozyme markers, and the geographic range of population structure was estimated at 100 km (Kotoulas et al. 1995). Other studies observed high gene flow in the North-East Atlantic using allozyme or Exon-Primed Intron-Crossing (EPIC) markers, without detecting an isolation-by-distance pattern (Exadactylos et al. 1998; Rolland et al. 2007). Genetic differences between continental Europe (Bay of Biscay and German Bight) and the British Isles (Eastern Coast of England and Irish Sea) were only found in a study using randomly amplified polymorphic DNA (RAPD) on a limited number of samples (Exadactylos et al. 2003). In general, the Atlantic populations are considered a single panmictic unit (Rolland et al. 2007). Nevertheless, the above mentioned studies included only few sampling locations from the North Sea and only one study included a sample from

the Kattegat (Rolland et al. 2007). Almost none of the samples were taken in the spawning season. Moreover, temporal stability of the genetic signal was investigated in only two studies, on a local scale in the Bay of Biscay (Kotoulas et al. 1995; Rolland et al. 2007). Therefore, these findings could be strengthened and supplemented by including more spatial samples from the northernmost distribution range of sole, using highly polymorphic markers, an increased sample size and temporal replicates.

The main purpose of this study was to elucidate the genetic population structure of sole in the North-East Atlantic Ocean, with a focus on the North Sea. The combined use of microsatellite markers and a mitochondrial marker provide the opportunity to examine the impact of contemporary processes (e.g. isolation-by-distance) versus historical events (e.g. range expansions). We further investigated the extent of juvenile dispersal by comparing genetic variation and differentiation of juvenile sole with adult individuals. Because temporal replicates were available for most of the North Sea samples, the temporal stability of the genetic signal was also examined.

MATERIALS AND METHODS

Biological samples

A total of 1499 adult sole (*Solea solea* L.) were caught at 17 locations across the North Sea, Irish Sea, Celtic Sea, Bay of Biscay, English Channel, Kattegat, Skagerrak and Belt Sea, during the period 2006-2008 (Fig.1). During the same period, 312 juvenile sole were sampled at four nursery grounds: the Wadden Sea near Texel, the Scheldt estuary near Zandvliet, off the Thames and along the Belgian coast (Table 1). Adult fish were caught by commercial vessels or during research surveys. Most adult sole were sampled in spring or summer, except for the samples from the Kattegat, Skagerrak and Belt Sea, which were sampled during an autumn survey. Juvenile fish were caught in autumn with fyke nets or beam trawl. Temporal replicates were available for six sampling locations within the North Sea to test for temporal stability between years or between seasons. Sample codes refer to the sampling location and sampling year (Table 1). Fish were either immediately stored frozen or measurements were done onboard and a fin tissue sample was preserved in 96 % ethanol.

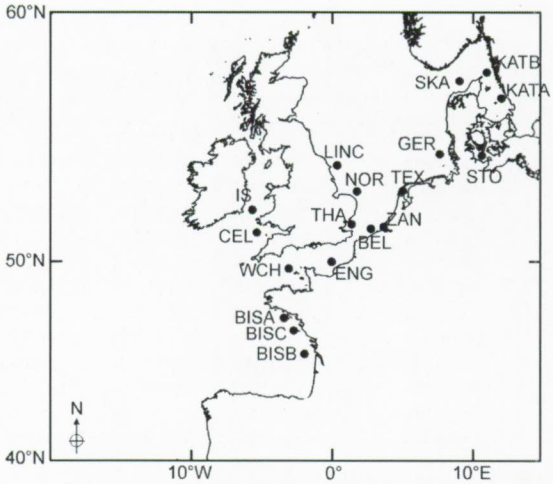

Fig.1 *Solea solea.* Map of study area with sampling locations indicated. See Table 1 for sample codes

Molecular methods

Total genomic DNA was isolated from a piece of pectoral fin tissue using the Nucleospin Tissue extraction Kit according to the manufacturer's conditions (Macherey-Nagel GmBH, Düren, Germany). Samples were initially genotyped for 15 microsatellite loci (F8-ICA9, F8-ITG11, F13-II8/4/7, F8-IIGT15, F14-IIGT16 (Iyengar et al. 2000); (Sos(AC)6, Sos(AC)20, Sos(AC)40, Sos(AC)30, Sos(AC)45, (Garoia et al. 2006); SolCA13, SolGA12, Sol19A (Porta & Alvarez 2004); SseGATA26, SseCA28 (Funes et al. 2004)) on an automated capillary sequencer ABI 3130 AVANT (Applied Biosystems). MtDNA data have been generated for 23 out of 28 samples; a total of 651 individuals were sequenced at a 590 bp fragment of the mitochondrial cytochrome *b* (cyt *b*) locus (Table 1). The cyt *b* gene was amplified by PCR using the primers CB1bis-F (5'-TACGTCCTCCCCTGAGGACAGATATC-3') and SolCytb1-R (5'-GGCGCTCTAACACTGAGCTAC-3'). Products were sequenced with the Forward primer only because of the unambiguous sequence results. Sequences of 590 bp were checked with SeqScape v. 2.1 (Applied Biosystems) and aligned with BIOEdit v.7.0.5 (Hall 1999). Details on the PCR conditions of microsatellite and mitochondrial markers are presented in the Supplementary Materials (Table S1).

Table 1 Sampling information for *Solea solea* including sample code, life stage (A=adult; J=juvenile), Location (see Fig. 1), Area, Sampling date (including attribution to the spawning season *), Latitude, Longitude, number of samples (N), number genotyped (G), number sequenced (S)

Sample	A/J	Location	Area	Date	Lat	Long	N	G	S
STO07	A	STO	Belt Sea	Oct/2007	55°10'29"N	11°02'44"E	48	45	0
KATA07	A	KATA	Kattegat	Nov/2007	57°08'91"N	11°38'52"E	48	44	0
KATB07	A	KATB	Kattegat	Nov/2007	56°25'31"N	12°11'21"E	48	44	39
SKA07	A	SKA	Skagerrak	Nov/2007	58°09'43"N	9°30'32"E	48	39	32
GER07	A	GER	North Sea	May/2007*	54°31'12"N	7°53'23"E	60	54	31
LINC07	A	LINC	North Sea	Aug/2007	53°19'96"N	0°25'63"E	96	47	0
LINC08	A	LINC	North Sea	Aug/2008	53°19'96"N	0°25'63"E	52	51	0
NOR07	A	NOR	North Sea	Aug/2007	53°00'70"N	1°33'62"E	46	45	14
NOR08	A	NOR	North Sea	Aug/2008	53°00'70"N	1°33'62"E	28	23	23
THA07	A	THA	North Sea	Aug/2007	51°27'80"N	1°20'00"E	96	94	17
THA08	A	THA	North Sea	Aug/2008	51°27'80"N	1°20'00"E	63	58	57
BEL07s	A	BEL	North Sea	May/2007*	51°23'22"N	3°10'01"E	96	96	10
BEL07f	A	BEL	North Sea	Aug/2007	51°21'14"N	2°55'45"E	80	75	3
BEL08s	A	BEL	North Sea	May/2008*	51°23'22"N	3°10'01"E	96	71	45
BEL08f	A	BEL	North Sea	Aug/2008	51°21'14"N	2°55'45"E	60	58	52
CEL08	A	CEL	Celtic Sea	Apr/2008*	50°49'00"N	5°01'00"W	96	81	44
IS08	A	IS	Irish Sea	Mar/2008*	52°13'00"N	5°20'00"W	96	91	44
ENG08	A	ENG	English Channel	Jul/2008	50°46'54"N	1°29'04"E	58	52	48
WCH09	A	WCH	English Channel	Aug/2009	49°39'41"N	2°07'38"W	80	37	0
BISA07	A	BISA	Atlantic Ocean	Mar/2007*	46°53'00"N	2°47'00"W	95	88	3
BISB07	A	BISB	Atlantic Ocean	Mar/2007*	45°36'00"N	1°24'00"W	61	55	5
BISC07	A	BISC	Atlantic Ocean	Mar/2007*	46°20'00"N	1°53'00"W	48	45	26
TEX06	J	TEX	Wadden Sea	Aug/2006	52°58'11"N	4°56'35"E	58	46	21
TEX07	J	TEX	Wadden Sea	May/2007	52°58'11"N	4°56'35"E	48	43	48
THA07J	J	THA	North Sea	Aug/2007	51°27'80"N	1°20'00"E	35	34	17
ZAN06	J	ZAN	Scheldt estuary	Sep/2006	51°23'50"N	4°06'59"E	74	70	15
ZAN07	J	ZAN	Scheldt estuary	Oct/2007	51°23'50"N	4°06'59"E	61	61	30
BEL08J	J	BEL	North Sea	May/2008	51°23'22"N	3°10'01"E	36	32	27
Total							**1811**	**1579**	**651**

Statistical analyses

To identify potential genotyping errors in the microsatellite data (i.e. stuttering, large allele dropout or null alleles), the software MICROCHECKER v.2.2.3 (van Oosterhout et al. 2006) was used. Null allele frequencies were also estimated for each locus and population using the Expectation Maximization algorithm of Dempster et al. (1977), carried out by the software FREENA (Chapuis & Estoup 2007). The software LOSITAN (Antao et al. 2008), based on the fdist F_{ST} outlier method of Beaumont & Nichols (1996) was used to assess if any of the microsatellite loci deviated from selective neutrality. A total of 95 000 simulations were computed for SMM with the options 'neutral mean F_{ST}' and 'force mean F_{ST}'. Deviations from Hardy-Weinberg equilibrium were tested per locus and sample using exact tests (Guo & Thompson 1992) implemented in GENEPOP v.2.3.4 (Raymond & Rousset 1995).

The statistical significance was adjusted using Bonferroni correction to correct for multiple testing (Rice 1989).

Genetic diversity

For the microsatellite data, the observed and unbiased expected heterozygosity and the number of alleles were analyzed in GENETIX v. 4.05 (Belkhir et al. 2004). Single and multilocus F_{IS} (Weir & Cockerham 1984) and allelic richness (AR) per locus (El Mousadik & Petit 1996) were calculated with FSTAT v.2.9.3 (Goudet 1995).

Haplotype diversity (h), nucleotide diversity (Π) and their standard deviation, and the number of polymorphic sites were calculated for the mtDNA using DNAsp v.5 (Librado & Rozas 2009).

Population structure

Genetic differentiation was analyzed with several methods for the microsatellite data. First, global and pairwise F_{ST} (Weir & Cockerham 1984) were estimated using FSTAT. Global and pairwise R_{ST} (Slatkin 1995) were estimated with SPAGeDi v.1.3 (Hardy & Vekemans 2002). To test if allele size is informative with respect to genetic structuring, 10 000 permutations of alleles were carried out. Finally, the unbiased estimator of divergence *'*D_{est}*'* of Jost (2008) was also calculated with SMOGD (Crawford 2010) because it is expected to perform better for populations with high allelic diversity and with alleles that are alternatively fixed in different populations (Jost 2008). A Principal Component Analysis based on allele frequency data was performed with PCAGEN v.1.2 (Goudet 2004) to visualize the relationships among samples; 10 000 randomizations were performed to test for the significance of axes.

Genetic structure based on microsatellite data was further described using a Bayesian clustering method, implemented in the program STRUCTURE v.2.3 (Pritchard et al. 2000; Hubisz et al. 2009). This method estimates the number of genetic clusters in the data without making any a priori assumptions about population structure. The 'no-admixture' algorithm was used with information on sampling location included to assist the clustering, allowing for better performance for data with weak structure (Hubisz et al. 2009). Only adult samples were included to estimate the number of clusters in the data (K). For each simulation of K (K = 1-10), ten independent replicates were used. The most likely number of clusters given the data was selected by choosing K with the largest log-likelihood according

to Evanno et al. (2005). 10 000 runs were used as burn-in and 100 000 MCMC repeats after burn-in, assuming correlated allele frequencies among populations. The proportional coefficients (Q) of individuals from STRUCTURE were plotted with the software DISTRUCT 1.1 (Rosenberg 2004) to visualize the patterns in clustering of predefined populations.

For the mitochondrial sequences, population differentiation was estimated using global G_{ST} (Nei 1973) and with pairwise F_{ST} and genetic distance of Tamura and Nei (1993), with ARLEQUIN v. 3.11 (Excoffier et al. 2005). Jost's D estimator ('D_{est}') was calculated using samples with more than 20 sequences, with SPADE (Chao & Shen 2010). Intraspecific relationships among the haplotypes were visualized with a median joining network, implemented in the software NETWORK v. 4.5.1.6 (www.fluxus-engineering.com). A multidimensional scaling analysis (MDS) was conducted with STATISTICA v.9 (Statsoft) to visualize genetic relationships among samples. This analysis was done based on genetic distance of Tamura and Nei (1993). Stress values below 0.20 provide interpretable information regarding intersite relationships (Clarke 1993).

To compare the degree of population differentiation between both types of markers, the pairwise F_{ST} (Rousset 1997) matrix of the microsatellite data was correlated with the genetic distances of Tamura and Nei (1993) from the mtDNA, using a Mantel procedure (Mantel 1967) in Genetix. Significance was tested with 2000 permutations.

Isolation-by-distance

To test for a population differentiation pattern following an isolation-by-distance model, a Mantel test (Mantel 1967) with the log of geographical distance and $\theta/1\text{-}\theta$ for the microsatellite data and genetic distance for the mitochondrial data, was carried out in GENETIX. Only adult samples were included and for the sequence data only those samples with more than 20 sequences were included. The correlation was tested first including all samples and secondly, using only the samples along the European continental shoreline. Significance was tested with 2000 permutations. Geographical distance between sampling locations was calculated using Microsoft ENCARTA World Atlas 2001. Distances were measured as the shortest coastal distances between sampling locations because adult sole remain restricted to waters less than 50 m deep.

Temporal differentiation

To evaluate the degree of temporal variation we performed a hierarchical analysis of molecular variance in ARLEQUIN v.3.1 (Excoffier et al. 2005), comparing temporal replicates of the microsatellite data from the same geographical location (2007 samples versus 2008 samples). Significance levels were determined after 10100 permutations.

Juvenile dispersal

Genetic variation (AR, Ho, He, relatedness) and differentiation (F_{ST}) was compared between adult samples and juvenile samples in FSTAT and the confidence level tested using 10 000 permutations. To investigate if we could assign juvenile sole to one of the adult populations, an individual assignment test was done with the software GENECLASS (Piry et al. 2004) using the bayesian method of Rannala & Mountain (1997). For this analysis, we only included the following adjacent populations as baseline samples: Thames (THA07 + THA08), Norfolk Banks (NOR07 + NOR08), Belgian Coast (BEL07s + BEL08s), German Bight (GER07), Eastern English Channel (ENG08), Western English Channel (WCH09) and Skagerrak (SKA07).

Effective population size

Point estimates of short-term effective population size (N_e) were obtained for all samples with a linkage disequilibrium method using the software LDNe v.1.31 (Waples & Do 2008), correcting for downward bias due to small samples sizes relative to N_e. 95 % confidence intervals were estimated using a jack-knifing method.

RESULTS

The MICROCHECKER analysis indicated that loci F8-IIGT15, F14-IIGT16, Sos(AC)30 and Sos(AC)40 might be affected by null-alleles or stuttering in most samples. Therefore these loci were excluded from further statistical analyses. The average null allele frequency estimated by the EM algorithm in FREENA (Dempster et al. 1977) was lower than 10 % in each of the 10 remaining loci (Table 2). The test with LOSITAN confirmed selective neutrality for all 10 microsatellite loci. Significant deviations from Hardy-Weinberg equilibrium were found for 8 of the 280 tests conducted, after Bonferroni correction. Jackknifing over the loci did not alter the multilocus estimates of F_{IS} or F_{ST}.

Table 2 Null allele frequency per locus; single locus and multilocus F_{IS}, F_{ST}, R_{ST} and D_{est}. Significant values are listed in bold.

	Null freq.	F_{IS}	F_{ST}	R_{ST}	D_{est}
SolCA13	0.013	0.030	**0.006**	**0.017**	**0.033**
Sos(AC)20	0.009	0.020	0.001	**0.007**	**0.004**
Sos(AC)45	0.048	0.127	0.001	-0.001	**0.011**
F13-II8/4/7	0.016	0.027	0.004	-0.001	**0.010**
F8-ICA9	0.019	0.000	0.001	**0.011**	**0.002**
F8-ITG11	0.012	0.016	0.001	-0.003	**0.006**
SolGA12	0.022	0.056	< 0.0001	0.005	**0.000**
Sos(AC)6	0.057	0.135	**0.004**	0.001	**0.041**
SseCA28	0.011	0.025	0.002	-0.001	**0.009**
SseGATA26	0.011	-0.013	**0.005**	**0.010**	**0.012**
Multilocus	0.022	0.047	**0.002**	**0.005**	0.006

Genetic variability

All loci were polymorphic, with the number of alleles per microsatellite locus within samples varying from 3 at locus F8-ICA9 to 27 at locus Sos(AC)20 (Supplementary Materials,Table S2). The mean number of alleles per locus varied from 8.9 in Norfolk (NOR08) to 15.1 in the BEL07s sample. The lower number of alleles in NOR08 and BEL08j is most likely an artifact due to their lower sample size (N = 23, N = 32, respectively). This is confirmed by the mean allelic richness, a measure of diversity corrected for sample size. The mean allellic richness based on 20 individuals did not differ much among samples, ranging from 8.19 in Skagerrak (SKA07) to 9.51 in the Texel sample (TEX07) (Table 3). Average observed heterozygosities varied from 0.668 in the sample from Norfolk banks (NOR08) to 0.804 in the juvenile sole from the Belgian coast (BEL08j). Expected heterozygosity was uniformly high, ranging from 0.725 in NOR08 to 0.775 in the Thames sample of 2007 (THA07) (Table 3). Expected heterozygosity was slightly lower in the samples from the Baltic region (Kattegat, Skagerrak, Belt Sea; mean He = 0.74) compared with the expected heterozygosity of all North Sea samples (mean He = 0.76). F_{IS} values ranged from -0.06 in the sample from the Belgian coast juveniles (BEL08j) to 0.105 in the sample from the Belgian spawning and feeding population (BEL08s and BEL08f). Twenty one of the 28 samples showed significant F_{IS} values (Table 3).

Table 3 Estimates of genetic diversity of all samples based on 10 microsatellite markers. Non-biased expected heterozygosity (He), observed heterozygosity (Ho), mean number of alleles (MNA), allelic richness (AR, based on 20 individuals) and F_{IS} values. See Table 1 for sample codes.

	Sample Code	He	Ho	MNA	AR	F_{IS}
ADULTS	STO07	0.744	0.748	11.3	8.68	-0.004
	KATA07	0.743	0.706	10.9	8.29	**0.050**
	KATB07	0.743	0.761	10.9	8.33	-0.024
	SKA07	0.730	0.710	10.0	8.19	0.028
	GER07	0.746	0.703	11.8	8.55	**0.058**
	LINC07	0.767	0.727	11.8	9.09	**0.053**
	LINC08	0.761	0.732	12.6	9.23	**0.039**
	NOR07	0.772	0.722	11.4	8.88	**0.065**
	NOR08	0.725	0.668	8.9	8.51	**0.080**
	THA07	0.775	0.740	14.2	9.03	**0.045**
	THA08	0.747	0.739	12.7	8.95	0.011
	BEL07s	0.769	0.718	15.1	9.09	**0.067**
	BEL07f	0.767	0.710	12.9	8.83	**0.074**
	BEL08s	0.771	0.691	14.5	9.43	**0.105**
	BEL08f	0.771	0.691	12.5	9.07	**0.105**
	CEL08	0.748	0.741	13.7	8.73	0.009
	IS08	0.766	0.762	14.8	9.15	0.006
	ENG08	0.760	0.702	12.4	8.98	**0.077**
	WCH09	0.747	0.700	13.6	8.86	**0.064**
	BISA07	0.766	0.743	14.2	9.15	**0.030**
	BISB07	0.751	0.700	12.0	8.77	**0.068**
	BISC07	0.763	0.726	11.5	8.80	**0.049**
JUVENILES	TEX06	0.758	0.743	11.0	8.42	0.020
	TEX07	0.755	0.723	12.7	9.51	**0.043**
	THA07j	0.750	0.686	11.5	9.43	**0.086**
	ZAN06	0.756	0.706	12.9	8.88	**0.067**
	ZAN07	0.773	0.732	13.1	9.18	**0.054**
	BEL08j	0.753	0.804	9.4	8.43	**-0.069**

The 651 mtDNA sequences of 590 bp contained 79 variable sites with 46 parsimony informative sites and 33 singleton variable sites (see Supplementary Materials, Table S3 for details on variable nucleotide positions). The diversity was very high, with 107 haplotypes detected. Haplotype A2 was by far the most dominant haplotype, detected in 40 % of the samples. Haplotypes A3, A5, A8 and A14 were also shared between multiple sites, occurring in more than 20 % of the samples. There were 63 unique haplotypes found; 6 haplotypes occurred only in the Skagerrak/Kattegat region; 5 haplotypes were unique to the Bay of Biscay and 9 were unique to the Irish/Celtic Sea (Supplementary Materials, Table S4). The overall level of genetic variability was high, with haplotype diversity and nucleotide diversity

0.823 and 0.0049, respectively. The highest diversity was observed in the Skagerrak and the lowest in THA08 and BISC07 (not taking into account BISA07 with only 3 sequences) (Table 4).

Table 4 Molecular diversity indices of mtDNA sequences: sample code, number of sequences (N), number of polymorphic sites (S), number of haplotypes (h), haplotype diversity Hd (Standard Deviation) and nucleotide diversity Π (Standard Deviation). See Table 1 for sample codes.

	Sample code	N	S	h	Hd		Π	
ADULTS	KATB07	39	18	13	0.825	(0.051)	0.0061	(0.00059)
	SKA07	32	21	17	0.950	(0.019)	0.0066	(0.00056)
	GER07	31	18	13	0.815	(0.066)	0.0044	(0.00081)
	NOR07	14	18	12	0.967	(0.044)	0.0064	(0.00114)
	NOR08	23	15	11	0.806	(0.079)	0.0055	(0.00080)
	THA07	17	8	7	0.824	(0.064)	0.0032	(0.00046)
	THA08	57	24	19	0.751	(0.060)	0.0043	(0.00062)
	BEL07s	10	12	6	0.867	(0.085)	0.0069	(0.00090)
	BEL07f	3	7	3	1.000	(0.272)	0.0079	(0.00324)
	BEL08s	45	25	20	0.782	(0.065)	0.0047	(0.00067)
	BEL08f	52	30	25	0.853	(0.044)	0.0055	(0.00051)
	BEL08j	27	13	13	0.840	(0.059)	0.0039	(0.00075)
	CEL08	44	17	16	0.813	(0.053)	0.0031	(0.00046)
	IS08	44	21	21	0.794	(0.064)	0.0048	(0.00063)
	ENG08	48	24	18	0.778	(0.060)	0.0053	(0.00064)
	BISA07	3	6	2	0.667	(0.314)	0.0068	(0.00320)
	BISB07	5	5	4	0.900	(0.161)	0.0041	(0.00117)
	BISC07	26	14	12	0.754	(0.090)	0.0041	(0.00073)
JUVENILES	TEX06	21	14	11	0.819	(0.082)	0.0046	(0.00084)
	TEX07	48	20	16	0.795	(0.056)	0.0045	(0.00062)
	THA07j	17	13	10	0.875	(0.070)	0.0036	(0.00085)
	ZAN06	15	13	9	0.886	(0.069)	0.0053	(0.00091)
	ZAN07	30	19	16	0.871	(0.051)	0.0049	(0.00077)
	TOTAL data set	651	79	107	0.823	(0.015)	0.0049	(0.00017)

Population structure

The Principal Component Analysis based on microsatellite data showed three groups (Fig.2). The first group consisted of the samples of Kattegat, Skagerrak and the Belt Sea. The second group contained the North Sea samples, also including Irish and Celtic Sea and English Channel, but located at the edge of the cluster. The third group clustered together the samples from the Bay of Biscay.

The first axis explained 14.97 % of the genetic variation (F_{ST} = 0.002; p = 0.005) and separated group 1 and group 3 entirely. The second PCA axis explained 9.57 % of the total genetic variation (F_{ST} = 0.001; p = 0.546). The sample from Norfolk 2008 is also separated from the rest of the North Sea samples, although this might be an artefact resulting from its small sample size.

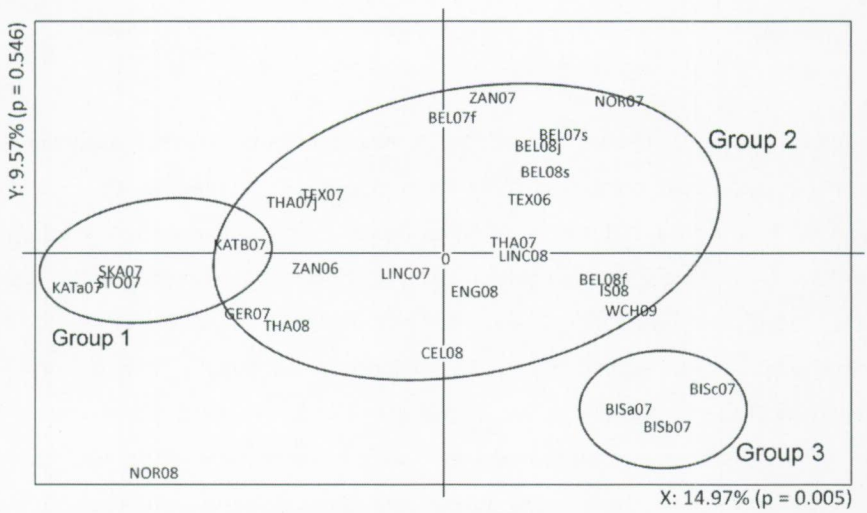

Fig.2 Principal Component Analysis based on microsatellite data of adult and juvenile samples. Sample codes (last two digits refer to sampling year): SKA: Skagerrak, STO: Belt Sea, KATa/KATB: Kattegat, THA: Thames, NOR: Norfolk Banks, GER: German Bight, BEL: Belgian Coast, IS: Irish Sea, CEL: Celtic Sea, ENG: Eastern English Channel, WCH: Western English Channel, LINC: Lincolnshire, BIS: Biscay, TEX: Texel, ZAN: Scheldt. For more information, see Table 1.

The global F_{ST} based on the microsatellite data was very low 0.0024 (95% C.I.: 0.001-0.003) but highly significant (p < 0.0001). Significant spatial differentiation based on pairwise F_{ST} values was observed between the samples from the most Northern region (Skagerrak, Kattegat and Belt Sea) and the rest of the samples. Also the Celtic Sea was genetically different from most of the samples with pairwise F_{ST} values up to 0.004. The spawning populations of the Irish and Celtic Sea in this study were marginally differentiated (F_{ST} = 0.005; p = 0.05). Within the North Sea, patterns are less clear, with temporal instability and several genetic differences that did not remain significant after Bonferroni correction. Especially the sample from Norfolk banks (2007) showed significant differentiation from other North Sea samples such as the German Bight, Thames and Belgian Coast. The Bay of Biscay samples differed from the Baltic region and from many of the North Sea samples.

Only the genetic differences between NOR07 and KATA07 (F_{ST} = 0.001) and between BEL07f and BISA07 (F_{ST} = 0.004) remained significant after Bonferroni correction (Supplementary Materials, Table S5). The global R_{ST} was 0.0044 ($p < 0.0001$). Pairwise R_{ST} values ranged from -0.01 to 0.07 (Supplementary Materials, Table S5).

The harmonic mean of Jost D_{est} was 0.006 and pairwise D_{est} were generally slightly higher than F_{ST} estimates but showed similar patterns (Supplementary Materials, Table S6).

The clustering approach in STRUCTURE resulted in the assignment of the individuals to one of three hypothetical clusters (highest ΔK for K = 3) (Fig. 3, Fig. 4). The lowest proportion of assignment to a particular cluster was 0.43 to cluster 2 for the sample from the German Bight (GER07). An equally large proportion (0.43) was assigned to cluster 3 for this specific sample. The highest proportion of assignment to a cluster was 0.887 for the Western Channel sample to the second cluster. The majority of the samples from the Belt Sea, Kattegat and Skagerrak had the highest assignment value for the third cluster. Most of the other individuals from the North Sea samples showed highest assignment to the first or the second cluster. Although there are no obvious population assignments to cluster 1, visual inspection suggests that samples from the Bay of Biscay differ slightly from samples from the North Sea (Fig. 4). The western English Channel seems more affiliated with the Biscay cluster, while the Eastern English Channel is most similar to the North Sea cluster. The Irish and Celtic Sea are very similar to samples from the North Sea. When we forced K = 2, the samples from the Belt Sea, the Kattegat and the Skagerrak clustered together, while all other samples showed the highest probability for the other cluster. The German Bight consisted of a mixture of both clusters (Fig. 3, Fig. 4). Leaving out the samples of the Baltic region did not improve the spatial resolution within the North Sea region (data not shown).

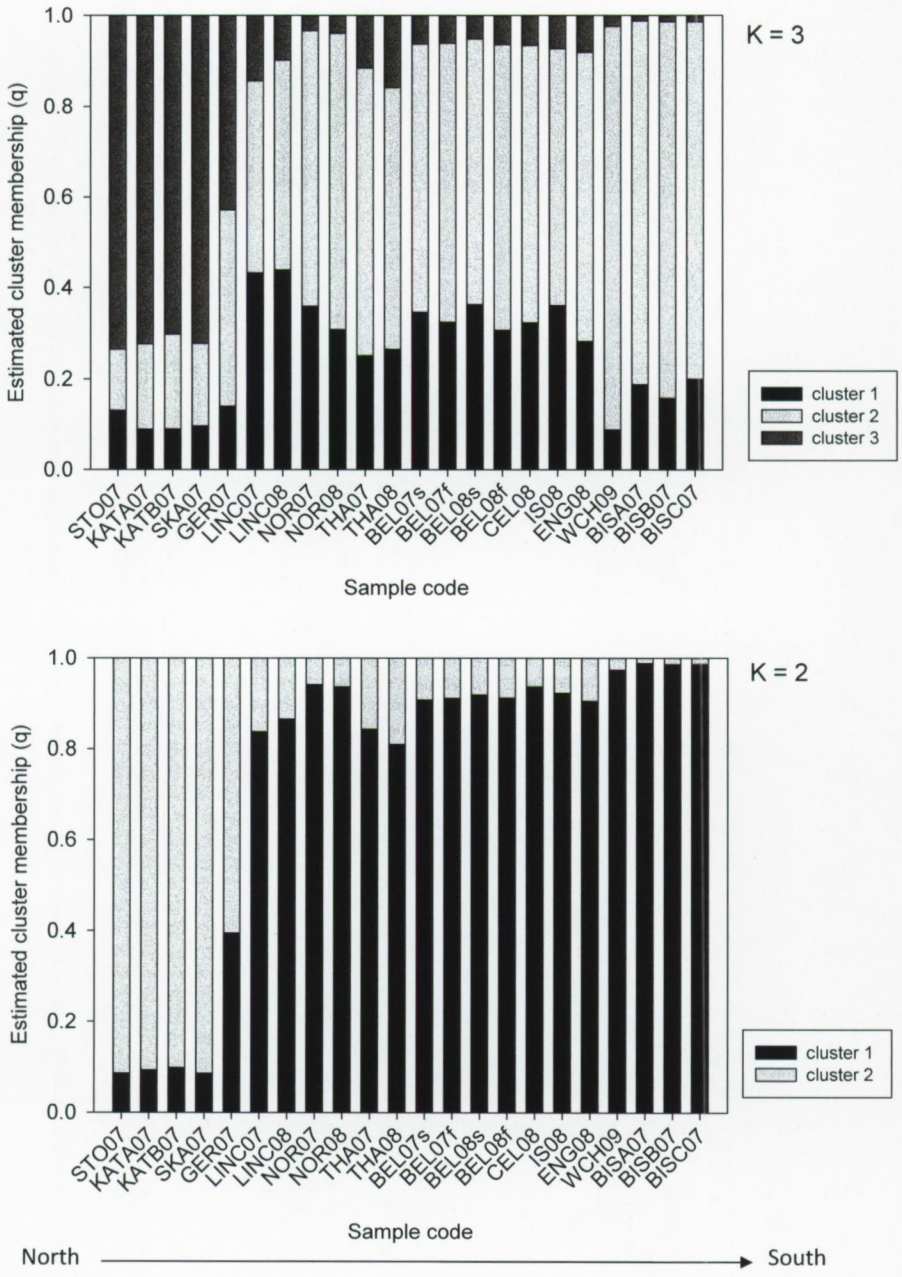

Fig. 3 *Solea solea*. Estimated probability of cluster membership for each sample, based on microsatellite data from STRUCTURE (K=3, upper graph; K=2, lower graph). X-axis indicates samples codes (See Table 1).

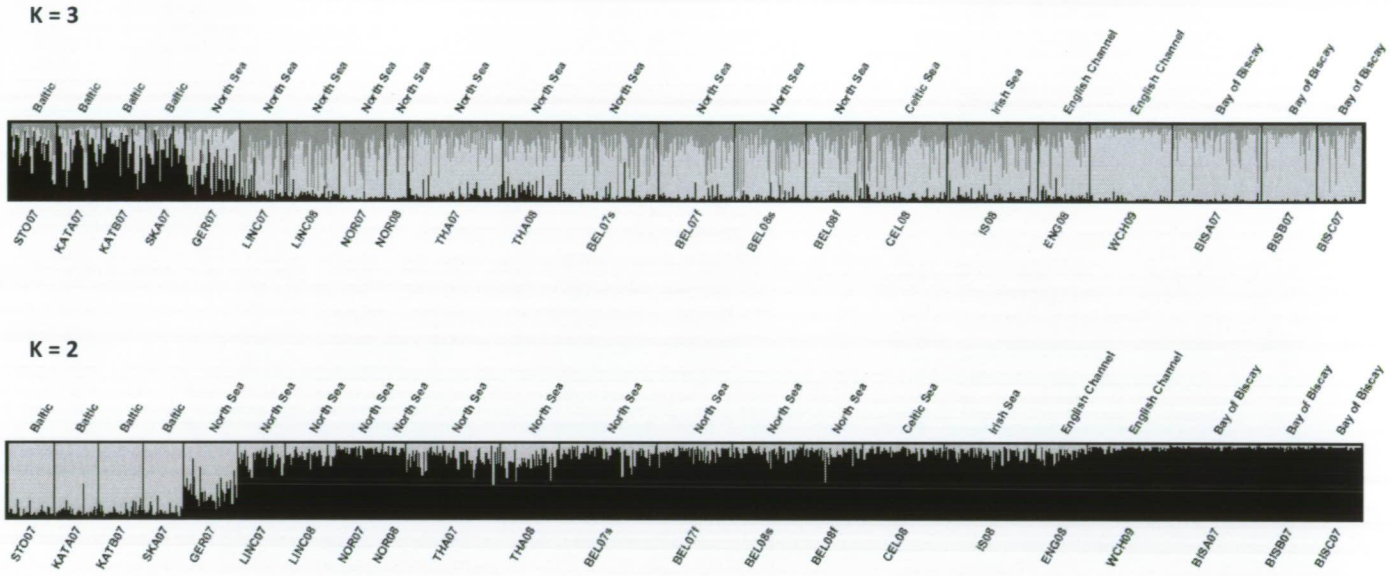

Fig. 4 *Solea solea.* Individual assignment based on Bayesian clustering method based on microsatellite data from STRUCTURE. Each bar represents an individual with its probability of membership to one of the hypothetical clusters (K=3, upper graph; K=2, lower graph). Labels on top indicate sampling regions; labels at the bottom indicate samples codes. See Table 1 for more information on samples.

MtDNA showed similar patterns of spatial differentiation but higher absolute values with G_{ST} = 0.0037 and pairwise F_{ST} values ranging from -0.020 between Eastern Channel and Norfolk Banks to 0.089 between the Kattegat and Celtic Sea. Spatial differentiation was most obvious between the samples from Skagerrak/Kattegat and all other samples. Also the Celtic Sea was genetically distinct, with 6 out of 22 significant pairwise F_{ST} comparisons (Supplementary Materials, Table S7). Differentiation remained significant after Bonferroni correction for the comparisons of SKA07 and KATB07 with CEL08.

The haplotype network based on cyt *b* illustrates the large number of haplotypes with only one mutation difference from the central haplotype (Supplementary Materials, Fig. S1). Besides one central haplotype, there are three other haplotypes present in high frequency, with many derived haplotypes. There are no distinct groupings of samples.

The multidimensional scaling analysis based on mitochondrial data also showed the clear separation of the Kattegat/Skagerrak samples from the rest of the North Sea samples. The Celtic and Irish Sea were also separated from the other samples. The stress value was 0.018 (Fig. 5).

The pairwise genetic distances of the mtDNA data were not correlated with the genetic distances of the microsatellite data (Mantel r = 0.026, p = 0.384).

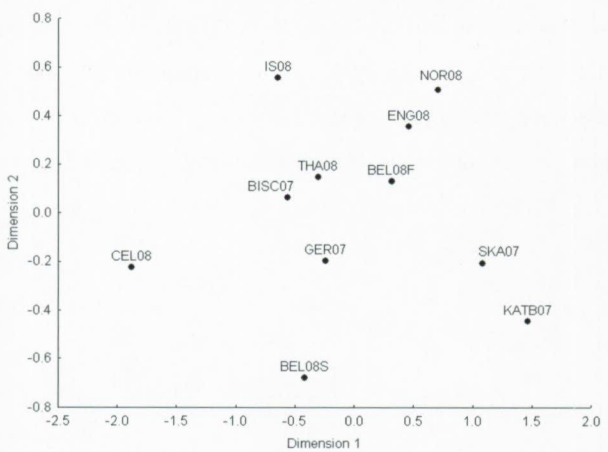

Fig. 5 Multidimensional Scaling plot based on pairwise genetic distance (Tamura-Nei, 1993) of mitochondrial data of sole. See Table 1 for sample codes.

Isolation-by-distance

A strong and significant isolation-by-distance pattern was found with the microsatellite data including only the adult samples along the continental shoreline ($r = 0.697$; $p = 0.002$). If samples from the British Isles and Ireland were included, the positive correlation between geographic and genetic distance was only weak and not significant. As for the cyt *b* sequences, no significant pattern of isolation by distance could be detected, although a positive linear trend was obvious (all samples: $r = 0.307$; $p = 0.09$; only continental samples: $r = 0.298$, $p = 0.19$) (Fig. 6).

Temporal differentiation

The AMOVA analysis indicated that almost all genetic variation (99.9 %) was attributed to the 'within sample' component ($F_{ST} = 0.002$, $p = 0.01$), while the variation among sampling years was only 0.21 % and not significant ($F_{CT} = 0.0002$, $p = 0.93$). Except for the samples from Norfolk Banks (NOR07 and NOR08), no pairwise comparisons (F_{ST}) of temporally replicated samples from the same location were significant (Supplementary Materials, Table S5). Pairwise F_{ST} among the temporal replicates ranged from -0.0002 between the feeding samples of the Belgian Coast (BEL07f vs. BEL08f) to 0.0117 between the temporal samples from the Norfolk Banks. The significant F_{ST} at Norfolk Banks ($F_{ST} = 0.0117$; $p = 0.01$) might be attributed to the low sample size in 2008 ($N = 23$). There was no significant genetic differentiation between the Belgian sole samples caught during the spawning season (BEL07s and BEL08s) and the samples caught during late summer of the same year (BEL07f and BEL08f) (Supplementary Materials, Table S5). Although insignificant, temporal F_{ST} values were of similar magnitude than spatial F_{ST} values.

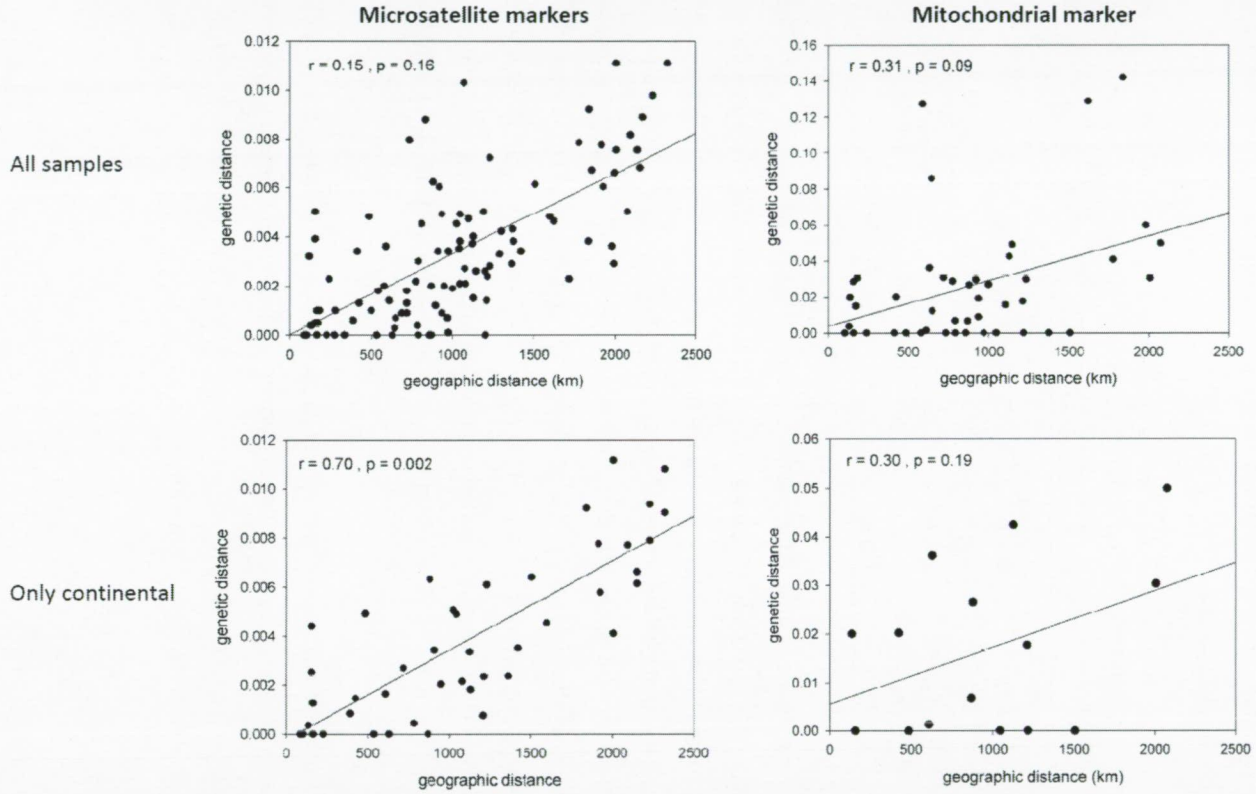

Fig.6 Relationship between genetic distance and geographic distance of sole for microsatellite markers (left panels) and for mitochondrial marker (right panels). Upper graphs include all samples, lower graphs include only samples along the continental shoreline. See text for more details.

Juvenile dispersal

Genetic variation in juvenile and adult samples was comparable and not significantly different (Table 5). There were no indications for reduced genetic variability or increase in relatedness in the juvenile samples.

Table 5 Comparison of genetic variation in adult and juvenile sole.
P-values after 1000 permutations

	Adults	Juveniles	p-value
Ho	0.721	0.720	0.920
Hs	0.757	0.760	0.630
F_{IS}	0.047	0.053	0.680
F_{ST}	0.002	0.002	0.470
REL	0.005	0.003	0.470
RELC	-0.099	-0.112	0.680

There was no significant overall differentiation among the juvenile samples from the different locations (F_{ST} = 0.0018; p = 0.058). Pairwise F_{ST} estimates ranged from -0.001 to 0.008. Some differentiation was apparent between the juveniles from Texel (both years) and the Belgian coast and between the Scheldt (ZAN06) and the Belgian Coast (BEL08j), but these differences did not remain significant after Bonferroni correction. There was no temporal differentiation present between the juvenile samples from the Wadden Sea (TEX) or from the Scheldt (ZAN) during two successive years (Supplementary Materials, Table S6). Based on the PCA plot, all juvenile samples are clustered within the 'North Sea' adult group. Temporal replicates of the same juvenile nursery location are not necessarily lying closely together on the graph (Fig. 2).

As expected from the low genetic differentiation between adult samples, the juvenile assignment test in GENECLASS resulted in almost random assignments of juveniles to the adult baselines (Table 6). Even if the baseline data were pooled into larger groups, there were no consistent regional patterns (data not shown).

Table 6 Assignment percentages of juveniles to adult baselines based on microsatellite analysis with GENECLASS. See Table 1 for sample codes.

		Assigned population						
		SKA	GER	NOR	THA	BEL	ENG	WCH
Juvenile sample	TEX06	15.2	15.2	8.7	15.2	26.1	10.9	8.7
	TEX07	4.7	2.3	20.9	14.0	37.2	11.6	9.3
	THA07j	17.6	0.0	17.6	14.7	29.4	11.8	8.8
	ZAN06	12.9	5.7	14.3	21.4	24.3	8.6	12.9
	ZAN07	6.6	11.5	11.5	13.1	36.1	13.1	8.2
	BEL08j	18.8	15.6	12.5	9.4	15.6	9.4	18.8

Effective population size

Estimates of N_e were high for all populations, except for the Danish Belt sample (STO07), situated at the limit of the distribution range, which had an estimate of 107 and a finite upper confidence limit of only 470 individuals. Almost all other samples showed infinitely high upper confidence intervals (Table 7).

Table 7 Estimates and 95% confidence intervals of effective population size (N_e) of all sole samples using a linkage disequilibrium method (Waples & Do 2008). See Table 1 for sample codes.

Sample	Ne	[95 % CI]		Sample	Ne	[95 % CI]	
STO07	107.2	[56	471.8]	BEL08f	955.8	[175	Infinite]
KATA07	118.8	[49.6	Infinite]	CEL08	Infinite	[251.8	Infinite]
KATB07	3830.5	[99.3	Infinite]	IS08	1507.1	[191.1	Infinite]
SKA07	168.2	[54.9	Infinite]	ENG08	Infinite	[186.1	Infinite]
GER07	291.6	[68.8	Infinite]	WCH09	Infinite	[218.8	Infinite]
LINC07	Infinite	[246.6	Infinite]	BISA07	Infinite	[270	Infinite]
LINC08	Infinite	[217	Infinite]	BISB07	136.2	[71.3	629.8]
NOR07	Infinite	[320.3	Infinite]	BISC07	245.1	[87.9	Infinite]
NOR08	Infinite	[133.1	Infinite]	TEX06	274.8	[65.6	Infinite]
THA07	Infinite	[328.4	Infinite]	TEX07	Infinite	[135.2	Infinite]
THA08	563.8	[95.6	Infinite]	THA07j	Infinite	[94	Infinite]
BEL07s	1006.6	[225.5	Infinite]	ZAN06	113.9	[56.6	580.2]
BEL07f	Infinite	[339.6	Infinite]	ZAN07	Infinite	[342.9	Infinite]
BEL08s	645.3	[146.2	Infinite]	BEL08j	Infinite	[747.2	Infinite]

Discussion

Overall, our study showed the clearest genetic divergence following an isolation-by-distance model at the largest scale, namely between sole populations from the Bay of Biscay and the Baltic transition region, and a more subtle genetic structure within the North Sea. This pattern was supported by the correlation between geographic and genetic distance based on microsatellite markers, and maintained by the samples from the Baltic region.

Population structure

In contrast to the population genetic study of sole by Rolland et al. (2007) which used three EPIC markers, we found strong and significant genetic differences between sole from the Baltic transition region (Skagerrak/Kattegat/Belt Sea) and the North Sea. This pattern was evident from all analyses, both with microsatellite and mitochondrial markers and is in accordance with the pattern observed for several other marine fish such as turbot (Nielsen et al. 2004), herring (Bekkevold et al. 2005; André et al. 2010), sprat (Limborg et al. 2009), plaice (Hoarau et al. 2004), hake (Lundy et al. 1999), cod (Nielsen et al. 2003) and sand goby (Larmuseau et al. 2010). The significant differentiation between the North Sea and the Baltic transition zone could be the result of environmental factors, biological factors or selection. The Skagerrak, Kattegat and Belt Sea provide a transition zone between the saline North Sea and the brackish Baltic proper. Based on the hydrodynamics of the area (Rodhe 1996), it is expected that larval exchange is mainly directed into the Skagerrak (Knutsen et al. 2004; Stenseth et al. 2006). For sole, this region represents the northeastern limit of its distribution, as the species is only occasionally present in the Baltic proper (Bacevičius & Karalius 2008). For many other taxa a loss of genetic diversity due to genetic drift is reported in this transition zone compared to populations in the Atlantic Ocean (Johannesson & Andre 2006). We observed an average expected heterozygosity of microsatellite markers slightly lower in sole from the Skagerrak and Kattegat compared to the North Sea samples, although mitochondrial haplotype diversity remained very high in this region. The area is recolonized after the Last Glacial Maximum (about 7-8 kya) from the North Sea and the high genetic diversity still found in this region may be due to the high effective population size, preventing loss of genetic diversity through genetic drift.

Allozyme data on sole indicated near panmixia between the North Sea, the Bay of Biscay and the Irish Coast populations and suggested probable movement of individuals through the English Channel (Exadactylos et al. 1998; Exadactylos et al. 2003). In our study, significant genetic differentiation based on microsatellite markers between the Bay of Biscay and the North Sea was apparent from the pairwise F_{ST} values, but less obvious from the Bayesian clustering approach. In contrast to earlier studies on sole using allozyme markers and RAPD markers, we also found small genetic differences between the Irish/Celtic Sea and some of the North Sea samples, although these differences were inconsistent between years and did not remain significant after correcting for multiple testing. The lack of strong differentiation between the Irish Sea and North Sea is also apparent for other North Atlantic fish like plaice (Was et al. 2010), turbot (Hemmer-Hansen et al. 2007b) and whiting (Charrier et al. 2007).

Finally, the very low genetic divergence among sole populations within the North Sea is also consistent with other marine fish (Borsa et al. 1997; Hoarau et al. 2002; Nielsen et al. 2004; Hemmer-Hansen et al. 2007b) and suggests high levels of gene flow. Gene flow could occur either through passive drift during the larval stage or through active migration of (sub)adults. On the one hand, passive mixing during the egg or larval stages might directly cause an exchange of genetic material from different spawning aggregations. Although a particle tracking model coupled to a hydrodynamic model has shown that the exchange of sole larvae among the main spawning sites is much lower than the number of larvae retained, some connections among spawning sites are possible with the level of exchange mainly depending on hydrodynamic variability (Savina et al. 2010). On the other hand, a limited number of active subadult or adult migrants between populations across the Channel would suffice to prevent strong genetic differentiation (Waples 1998). Although sole undertake relatively short migrations, active movements of tagged sole across the English Channel have been observed (Burt & Millner 2008). Average migration distances of mature fish were estimated at 75 km in the spawning season and up to 150 km during the rest of the year. The English Channel may thus act as a channel for gene flow between the British Isles and the southern North Sea through passive drift of larvae following the residual current flowing eastward or through active migration of (sub)adults.

Besides gene flow, weak population structure may also result from the recent colonization histories following postglacial sea level rise and high effective population size (N_e), reducing

the effects of genetic drift detected by microsatellite markers. For sole, effective population size estimates based on microsatellite markers were large and ranged to infinity. This implies that it will take a long time before the equilibrium F_{ST} is reached. Therefore low F_{ST} values do not necessarily imply high gene flow (Whitlock & McCauley 1999; Hedgecock et al. 2007; Larmuseau et al. 2010). Because mitochondrial markers have an effective size four times smaller than microsatellite markers, the effects of genetic drift appear faster, enabling the detection of population structure at an earlier level (Buonaccorsi et al. 2001). In populations with high effective population sizes, such as in sole, mitochondrial markers might therefore show higher resolution power. Nevertheless, even though the mitochondrial signal of differentiation (pairwise F_{ST}) was an order of magnitude higher than differentiation based on microsatellite makers, the spatial resolution power and general differentiation patterns were very similar in our study. No evidence of isolation-by-distance was found using the mitochondrial marker, but there was a positive trend observed between genetic distance and geographic distance. The limited resolution with the mitochondrial marker in our study could be due to the single-locus effect of the cyt *b*, compared to the use of multiple microsatellite loci.

Sampling design and temporal stability

Besides the aforementioned biological reasons for the observed genetic pattern, one should also be cautious when interpreting genetic results from highly dispersive marine taxa. Technical problems such as scoring errors and limited resolution power may reduce the signal of differentiation detected by microsatellite makers. We have carefully checked the genotypes and removed all markers with high frequency of null alleles. Still, estimates of population differentiation and the chances of detecting significant differentiation diminish when heterozygosity and allellic richness are high. This appears to be the consequence of size homoplasy at microsatellite markers (O'Reilly et al. 2004). Furthermore, sampling did not always occur within the spawning period. Even though the summer samples taken along the English coast were caught at spawning grounds, they could in theory present a mixed feeding aggregation. Nevertheless, the Belgian sole sampled in the spawning season in spring (BELs) was not significantly different from the sole sampled in late summer (BELf) and this was true for both 2007 and 2008, suggesting temporal stability of the spring and summer population along the Belgian Coast. None of the pairwise tests between temporal

samples taken from the same location in both years were significant, but temporal variation was of the same magnitude as spatial variation. If larval mixing indeed occurs in the North Sea, temporal variation between years might also be the result of differential larval dispersal patterns, reflecting hydrodynamic variation.

Juvenile dispersal

If larval dispersal is limited, the genetic structure of juveniles would reflect nearby adult spawning populations and show reduced genetic variation. However, we observed similar levels of genetic diversity in juvenile sole compared to adult fish. The absence of distinct genetic differentiation among juvenile samples from different nurseries could indicate either that adult fish form a single population or that juveniles arriving and settling at the nursery grounds are a mixture of progeny from different distinct populations. Since we could not detect strong genetic differentiation among adult samples within the North Sea, we believe the first hypothesis is more likely. Probably the genetic differentiation observed among adult samples within the North Sea is simply too low for an accurate assignment of juveniles (Hedgecock et al. 2007). Although no significant temporal differentiation was observed between juveniles from the same location in successive years, these samples did not always cluster together on the PCA plot, suggesting some temporal variation in genetic composition. Such temporal differences might arise due to chaotic genetic patchiness (Larson & Julian 1999; Pujolar et al. 2006). Also for juvenile sole in the Bay of Biscay (Guinand et al. 2008) and for juvenile plaice in the Irish Sea (Watts et al. 2004), with neutral genetic markers no genetic differentiation was found among nurseries.

Management implications

This study could not find a mismatch between the current management units of *Solea solea* in the North-East Atlantic and the biological units in the sense that there were no genetically distinct subpopulations that were managed together. On the contrary, the number of management units seems larger than the number of biological units. Based on the neutral genetic markers, there is only evidence for three or four management units: the Baltic region (Skagerrak, Kattegat and Belt Sea), the North Sea and English Channel, and the Bay of Biscay. Possibly, the Irish and Celtic Sea represents a separate management unit. However, the data presented here showed some genetic differentiation that was not temporally stable. Before being useful in fisheries management, the temporal stability of genetic structure should be

established and all samples should be composed of spawning adults. Furthermore, decisions on management units should be based on a combination of genetic data (from different markers) and ecological data (Florin & Hoglund 2008).

Even if neutral genetic differentiation is small in the presence of gene flow, local adaptation might still characterize the different sole aggregations. It is well established that in large populations differentiation at selective traits occurs faster than the effects of genetic drift (Hauser & Carvalho 2008; Nielsen et al. 2009a). Hence, genetic markers under selection might show more power to detect fine scale population structure. The evidence from the literature has been expanding steadily thanks to better genome coverage (Guinand et al. 2008; Larmuseau et al. 2009; André et al. 2010).

Conclusion

Sole populations in the North-East Atlantic Ocean are characterized by a weak isolation-by-distance pattern and genetic homogeneity within basins. Genetic differentiation was highest between the Skagerrak/Kattegat region and the rest of the North Atlantic. Some differentiation also existed between the North Sea and the Bay of Biscay and subtle differences between the North Sea and the Celtic Sea/Irish Sea. Juvenile genetic variability was similar to adult diversity and there were almost no spatial genetic differences among nursery grounds. We believe that dispersal of fish larvae away from local spawning aggregations may lead to a mixing of juveniles from different origins at nearby nursery grounds. Once settled, movement of juveniles is probably limited. The observed genetic structure seems to be relatively stable on a short temporal time scale. Further research, incorporating postlarval sampling and hydrodynamic modelling, might help to conclude which dispersal model is most appropriate for sole.

ACKNOWLEDGEMENTS

This study was linked to the WESTBANKS project, which is supported by the Belgian Science Policy (BELSPO; contract no. SD/BN/01A). We thank all scientists and fishermen who contributed to the collection of samples: E. Nielsen and colleagues (DTU-AQUA), M.L. Bégout and K. Mahé (IFREMER), L. Bolle (IMARES), H. van der Veer (NIOZ); U. Damm and N. Rohlf (ISH-BFAFI), ILVO-Fisheries, the crew of RV Zeeleeuw and RV Belgica, E. Diopere, K. Vancampenhout, S. Geldof, J. Guelinckx (K.U.Leuven). E.C. acknowledges a grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). G.E.M. is a post-doctoral researcher funded by the Fund for Scientific Research (FWO Vlaanderen). M.H.D.L. is a post-doctoral researcher funded by the KULEUVEN (BOF PDM-short).

SUPPLEMENTARY MATERIALS

Table S1. Multiplex conditions for *Solea solea* microsatellites: primer concentration and annealing temperature.

Multiplex	Locus	GenBank Accession No.	Conc (µM)	Ta
1	Sos(AC)40	AY950592.1	0.1	
1	Sos(AC)45	AY950593.1	0.1	59°C to 56°C with -1°/cycle: 4 x
1	Sos(AC)30	AY950589.1	0.1	56°C: 34 x
1	SolCA13	AF441390.1	0.1	
1	F8-IIGT15	AF173852.1	0.05	
1	Sos(AC)20	AY950591.1	0.1	
1	Sol19A	AF441387	0.025	
2	F13-II8/4/7	AF173849.1	0.4	
2	SolGA12	AF441391.1	0.1	67°C to 57°C with -1°C/cycle: 11›
2	F8-ITG11	AF173855.1	0.1	57°C: 19 x
2	Sos(AC)6	AY950588.1	0.4	
2	SseCA28	AB177540.1	0.025	
3	F8-ICA9	AF173851.1	0.2	60°C to 54°C with -1°C/cycle: 7x
3	F14-IITG16	AF173853.1	0.1	54°C: 29 x
3	SseGATA26	AB177537.1	0.2	

PCRs were carried out in 10 µL volumes, using a Multiplex PCR Kit (Qiagen), forward and reverse primers and 1 µL of template DNA. All PCRs followed a touchdown protocol. PCR conditions were as follows: 95 °C for 15 min, 95 °C for 30 s, Ta (Table) for 90 s, 72 °C for 60 s, 95 °C for 30 s, Ta (Table) for 90 s, 72 °C for 60 s, 60 °C for 30 min followed by storage at 4 °C.

Mt DNA amplification and sequencing

The PCR was carried out in a total volume of 25 µl, containing 1 µl of genomic DNA, 1 x PCR buffer, 0.2 mM dNTPs, 0.8 µM of each primer, 2.5 mM of $MgCl_2$, 0.5 U of Taq DNA polymerase (Silverstar, Eurogentec, Seraing, Belgium) and mQ-H_2O. The PCR cycle was: 4 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C; with a final 7 min extension period at 72 °C. All PCR products were visualized on agarose gels with ethidium bromide. After purification with NUCLEOFAST (Macherey-Nagel), the PCR products were sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit according to the manufacturer's protocol on an ABI 3130 automated capillary DNA sequencer (Applied Biosystems).

Table S2. *Solea solea*. Estimates of genetic diversity per population: Number of samples (N), Number of Alleles (NA), Allellic richness (AR), non-biased expected heterozygosity (He), Observed heterozygosity (Ho), F_{IS} values. Significant F_{IS} values are in bold. (Part I)

	STO07	KATA07	KATB07	SKA07	GER07	LINC07	LINC08	NOR07	NOR08	THA07	THA08	BEL07s	BEL07f	BEL08s	BEL08f
SolCA13															
N	45	44	42	31	46	45	51	45	21	90	42	88	74	67	58
NA	11	9	9	7	11	10	11	10	7	10	10	11	10	12	9
AR	8.83	7.59	8.09	6.63	8.80	8.71	8.88	8.24	7.00	7.99	8.60	7.83	7.74	8.46	8.37
He	0.834	0.812	0.831	0.737	0.853	0.843	0.828	0.812	0.844	0.821	0.841	0.813	0.816	0.828	0.842
Ho	0.867	0.750	0.881	0.742	0.848	0.800	0.726	0.733	0.762	0.767	0.857	0.796	0.770	0.761	0.776
F_{IS}	-0.030	0.077	-0.053	-0.007	0.012	0.051	**0.125**	0.097	0.100	0.067	-0.020	0.022	0.056	0.081	0.066
Sos(AC)20															
N	45	44	44	38	48	45	51	45	22	90	42	91	74	65	58
NA	18	17	18	19	16	17	22	17	12	24	20	24	22	25	20
AR	12.15	10.73	12.29	13.74	10.81	11.53	13.05	12.23	11.43	12.19	12.49	13.01	12.31	14.39	13.40
He	0.798	0.749	0.801	0.808	0.792	0.789	0.816	0.844	0.747	0.801	0.779	0.805	0.801	0.838	0.854
Ho	0.711	0.796	0.864	0.816	0.792	0.756	0.804	0.822	0.682	0.733	0.881	0.791	0.784	0.769	0.759
F_{IS}	0.092	-0.063	-0.041	-0.010	0.004	0.043	0.015	0.026	0.090	**0.085**	-0.133	0.022	0.022	0.083	**0.092**
Sos(AC)45															
N	45	43	38	36	40	45	51	45	21	91	42	87	71	67	58
NA	12	10	10	10	12	14	12	10	7	12	13	13	11	12	10
AR	9.83	9.36	8.93	8.91	10.18	10.10	9.85	8.95	7.00	9.41	10.34	8.94	9.08	8.64	8.84
He	0.870	0.874	0.863	0.865	0.886	0.882	0.883	0.871	0.866	0.871	0.876	0.866	0.867	0.854	0.870
Ho	0.800	0.674	0.790	0.778	0.750	0.844	0.804	0.800	0.714	0.846	0.714	0.816	0.761	0.716	0.741
F_{IS}	0.093	**0.379**	**0.211**	0.102	**0.135**	0.043	0.090	0.082	0.179	0.029	**0.187**	0.058	**0.123**	**0.162**	0.155
F13-II8/4/7															
N	44	44	44	39	54	47	49	45	23	94	56	96	75	71	57
NA	12	12	6	9	11	13	10	9	10	17	11	15	13	14	11
AR	8.77	9.09	5.30	7.19	7.04	9.07	6.70	6.83	9.43	8.90	7.23	8.45	8.64	7.96	8.04
He	0.780	0.757	0.707	0.704	0.711	0.752	0.640	0.704	0.728	0.749	0.694	0.760	0.757	0.715	0.727
Ho	0.864	0.750	0.886	0.795	0.722	0.596	0.694	0.600	0.609	0.723	0.679	0.646	0.653	0.761	0.667
F_{IS}	-0.094	0.009	**-0.252**	-0.131	-0.010	**0.209**	-0.085	0.149	0.166	0.034	0.022	**0.150**	**0.138**	-0.064	0.080

Table S2. Part II

	CEL08	IS08	ENG08	WCH09	BISA07	BISB07	BISC07	TEX06	TEX07	THA07j	ZAN06	ZAN07	BEL08j
SolCA13													
N	73	91	51	70	76	44	42	41	41	34	58	61	20
NA	10	12	10	10	9	9	9	8	11	9	10	9	6
AR	7.21	8.05	8.62	8347	8.22	7.80	7.79	7.29	8.91	7.88	8.13	7.94	6.00
He	0.802	0.808	0.837	0.837	0.835	0.802	0.798	0.819	0.798	0.795	0.824	0.812	0.774
Ho	0.781	0.813	0.784	0.784	0.882	0.750	0.833	0.781	0.732	0.735	0.810	0.869	0.750
F_{IS}	0.027	-0.007	0.064	0.013	-0.056	0.065	-0.046	0.048	0.084	0.076	0.035	-0.071	0.032
Sos(AC)20													
N	78	91	51	77	75	47	45	44	41	34	66	61	22
NA	23	27	20	24	21	21	20	17	22	22	17	23	15
AR	13.26	13.88	12.72	12499	11.36	12.87	13.58	11.64	14.01	16.42	10.54	14.44	14.25
He	0.814	0.843	0.806	0.806	0.800	0.791	0.832	0.802	0.798	0.871	0.759	0.856	0.851
Ho	0.769	0.857	0.804	0.804	0.787	0.809	0.711	0.750	0.854	0.882	0.727	0.885	0.864
F_{IS}	0.055	-0.017	0.002	-0.002	0.016	-0.023	**0.147**	0.065	-0.071	-0.013	0.034	-0.035	-0.015
Sos(AC)45													
N	75	91	51	75	75	46	44	44	41	34	67	61	20
NA	11	12	13	14	14	10	10	12	12	11	12	15	10
AR	8.60	8.90	10.10	9886	9.85	8.99	8.71	9.69	10.20	9.66	9.41	10.63	10.00
He	0.848	0.866	0.882	0.882	0.863	0.869	0.861	0.860	0.881	0.850	0.863	0.879	0.821
Ho	0.720	0.725	0.765	0.765	0.800	0.761	0.818	0.818	0.829	0.677	0.791	0.672	0.750
F_{IS}	**0.152**	**0.163**	**0.134**	**0.205**	0.074	**0.125**	0.050	0.049	0.059	**0.207**	0.079	0.237	0.088
F13-II8/4/7													
N	80	91	52	80	88	55	45	42	43	34	69	59	31
NA	11	12	10	11	13	9	10	8	10	11	12	11	8
AR	7.34	7.83	6.89	6960	7.71	6.34	6.56	5.63	8.04	9.15	8.18	7.80	6.80
He	0.701	0.711	0.665	0.665	0.678	0.633	0.626	0.677	0.742	0.750	0.724	0.742	0.680
Ho	0.688	0.758	0.635	0.635	0.636	0.564	0.622	0.667	0.698	0.765	0.652	0.695	0.710
F_{IS}	0.019	-0.067	0.046	-0.066	0.062	0.111	0.006	0.015	0.060	-0.020	0.094	0.064	-0.044

Table S2. Part III

	STO07	KATA07	KATB07	SKA07	GER07	LINC07	LINC08	NOR07	NOR08	THA07	THA08	BEL07s	BEL07f	BEL08s	BEL08f
F8-ICA9															
N	43	44	39	38	53	44	50	45	23	94	57	96	75	71	58
NA	3	4	4	3	4	7	4	6	5	7	4	5	6	7	7
AR	2.47	2.91	3.28	2.53	2.99	5.59	3.19	4.03	4.61	4.12	2.70	3.22	3.94	4.24	3.95
He	0.311	0.461	0.520	0.372	0.464	0.539	0.471	0.507	0.398	0.497	0.400	0.469	0.481	0.515	0.437
Ho	0.326	0.546	0.590	0.395	0.472	0.409	0.480	0.511	0.348	0.489	0.456	0.510	0.413	0.409	0.362
F_{IS}	-0.084	-0.185	-0.099	-0.061	-0.025	**0.243**	-0.020	-0.008	0.129	0.015	-0.142	-0.088	0.141	**0.208**	**0.197**
F8-ITG11															
N	43	41	44	38	54	39	42	45	23	94	58	96	75	71	56
NA	9	10	12	7	9	8	11	11	9	11	10	13	12	10	12
AR	7.71	7.88	9.12	6.76	7.23	7.49	9.43	9.10	8.46	8.27	7.89	8.13	8.13	8.18	8.87
He	0.742	0.751	0.761	0.810	0.787	0.793	0.812	0.821	0.757	0.796	0.783	0.793	0.779	0.814	0.829
Ho	0.698	0.659	0.818	0.790	0.648	0.821	0.810	0.800	0.826	0.809	0.776	0.760	0.813	0.732	0.768
F_{IS}	0.045	0.124	-0.076	0.025	0.185	-0.036	0.003	0.026	-0.094	-0.016	0.009	0.041	-0.045	0.101	0.075
SolGA12															
N	45	42	44	36	52	31	49	45	23	94	58	96	75	66	56
NA	12	15	14	13	13	12	14	11	10	13	16	17	13	17	15
AR	9.71	11.90	9.77	10.52	9.61	10.71	11.78	9.14	9.68	9.28	11.73	9.89	10.20	11.65	10.70
He	0.804	0.861	0.748	0.820	0.788	0.814	0.849	0.801	0.816	0.802	0.861	0.801	0.844	0.830	0.844
Ho	0.778	0.881	0.750	0.667	0.731	0.774	0.857	0.778	0.783	0.745	0.862	0.760	0.733	0.727	0.786
F_{IS}	-0.031	-0.023	0.016	**0.189**	0.063	0.050	-0.010	0.029	0.042	0.072	-0.001	0.051	**0.132**	**0.125**	0.069
Sos(AC)6															
N	42	44	44	34	52	44	50	45	23	94	58	96	75	70	58
NA	14	10	14	13	14	14	12	16	12	15	15	19	16	15	15
AR	10.91	8.68	10.62	10.88	10.87	11.20	10.20	12.21	11.33	10.80	10.85	12.47	11.80	11.74	11.20
He	0.859	0.854	0.856	0.851	0.868	0.860	0.854	0.884	0.891	0.882	0.857	0.886	0.890	0.895	0.876
Ho	0.833	0.796	0.796	0.794	0.808	0.773	0.740	0.756	0.783	0.734	0.845	0.719	0.800	0.700	0.690
F_{IS}	0.020	0.070	0.049	0.068	0.072	0.103	**0.134**	**0.147**	0.124	**0.169**	0.014	**0.189**	**0.101**	**0.219**	**0.214**

Table S2. Part IV

	CEL08	IS08	ENG08	WCH09	BISA07	BISB07	BISC07	TEX06	TEX07	THA07j	ZAN06	ZAN07	BEL08j
F8-ICA9													
N	66	90	51	79	83	55	43	46	43	34	66	61	32
NA	6	8	7	7	8	5	6	4	6	3	6	6	4
AR	3.80	4.47	4.83	3599	5.11	3.57	4.62	3.85	3.86	2.59	3.75	3.68	3.49
He	0.438	0.510	0.481	0.481	0.512	0.435	0.499	0.513	0.442	0.442	0.500	0.480	0.538
Ho	0.530	0.567	0.471	0.471	0.530	0.346	0.581	0.565	0.395	0.412	0.439	0.393	0.813
F_{IS}	**-0.212**	-0.113	0.022	0.016	-0.035	0.207	-0.167	-0.103	0.106	0.069	0.128	0.182	**-0.522**
F8-ITG11													
N	80	82	47	65	87	55	45	46	42	34	68	61	32
NA	12	14	10	11	11	9	11	9	13	8	10	10	10
AR	8.58	9.33	7.90	7874	7.49	8.02	8.37	7.32	10.68	7.35	7.81	7.70	8.27
He	0.790	0.802	0.791	0.791	0.798	0.837	0.803	0.790	0.801	0.778	0.786	0.775	0.753
Ho	0.800	0.878	0.745	0.745	0.862	0.909	0.756	0.804	0.738	0.647	0.721	0.787	0.844
F_{IS}	-0.013	**-0.095**	0.060	0.119	-0.081	-0.087	0.059	-0.019	0.079	0.171	0.084	-0.015	-0.123
SolGA12													
N	80	86	52	80	83	55	45	42	43	34	66	61	30
NA	15	16	13	13	19	15	14	14	14	15	16	14	9
AR	10.09	10.01	9.47	10173	11.70	11.53	11.08	10.25	10.91	12.37	11.16	10.42	8.26
He	0.820	0.783	0.801	0.801	0.832	0.840	0.846	0.817	0.858	0.846	0.851	0.811	0.810
Ho	0.813	0.791	0.731	0.731	0.723	0.800	0.822	0.833	0.837	0.765	0.803	0.803	0.833
F_{IS}	0.009	-0.009	0.088	**0.204**	**0.132**	0.048	0.029	-0.020	0.024	0.097	0.056	0.010	-0.030
Sos(AC)6													
N	75	89	52	79	88	54	42	46	43	34	70	61	31
NA	19	16	13	15	16	16	11	14	13	15	16	15	11
AR	11.74	10.63	11.10	11035	11.76	11.90	9.58	11.08	11.08	12.47	11.55	10.66	10.07
He	0.879	0.873	0.893	0.893	0.861	0.873	0.851	0.844	0.880	0.894	0.873	0.876	0.875
Ho	0.827	0.730	0.731	0.731	0.648	0.685	0.643	0.739	0.837	0.765	0.714	0.803	0.839
F_{IS}	0.064	**0.164**	**0.183**	0.075	**0.249**	0.217	0.246	0.125	0.050	0.147	0.182	0.084	0.042

Table S2. Part V

	STO07	KATA07	KATB07	SKA07	GER07	LINC07	LINC08	NOR07	NOR08	THA07	THA08	BEL07s	BEL07f	BEL08s	BEL08f
Sseca28															
N	42	44	44	37	54	42	47	37	23	94	58	96	71	69	56
NA	12	14	14	13	18	18	17	10	12	17	16	19	15	15	18
AR	9.48	9.57	9.81	9.85	11.35	12.06	11.50	8.40	11.45	10.82	10.38	11.10	9.58	9.72	11.63
He	0.806	0.815	0.795	0.756	0.803	0.846	0.787	0.736	0.852	0.841	0.822	0.819	0.806	0.764	0.825
Ho	0.905	0.818	0.727	0.838	0.778	0.881	0.766	0.676	0.870	0.872	0.810	0.750	0.761	0.667	0.768
F_{IS}	-0.101	-0.004	0.065	-0.109	0.029	-0.042	0.026	0.083	-0.021	-0.038	0.015	**0.084**	0.057	**0.128**	0.069
Ssegata26															
N	42	43	40	37	46	39	50	43	23	94	57	94	75	69	58
NA	10	8	5	6	10	5	13	14	5	16	12	14	11	18	7
AR	6.06	5.25	4.48	4.86	6.36	4.42	7.76	9.72	4.73	8.51	7.32	7.69	6.90	9.37	5.41
He	0.569	0.510	0.523	0.576	0.521	0.554	0.672	0.740	0.346	0.686	0.561	0.672	0.628	0.660	0.599
Ho	0.571	0.535	0.625	0.487	0.457	0.615	0.640	0.744	0.304	0.681	0.509	0.628	0.613	0.667	0.586
F_{IS}	-0.005	-0.050	-0.186	0.157	0.082	-0.113	0.049	-0.006	0.123	0.008	0.094	0.066	0.023	-0.010	0.050

	CEL08	IS08	ENG08	WCH09	BISA07	BISB07	BISC07	TEX06	TEX07	THA07j	ZAN06	ZAN07	BEL08j
Sseca28													
N	78	76	52	77	84	55	44	41	43	33	67	61	29
NA	17	17	17	17	18	18	15	15	16	12	18	15	14
AR	9.99	10.96	11.54	10451	11.08	10.88	11.19	11.34	10.54	9.43	11.55	10.79	11.40
He	0.783	0.806	0.839	0.839	0.835	0.809	0.848	0.838	0.750	0.774	0.820	0.828	0.822
Ho	0.808	0.763	0.827	0.827	0.869	0.746	0.796	0.732	0.698	0.697	0.836	0.771	0.897
F_{IS}	-0.031	0.053	0.014	0.013	-0.041	0.079	0.062	**0.128**	0.070	0.101	-0.019	0.070	-0.093
Ssegata26													
N	60	90	51	77	81	52	43	46	41	33	69	58	31
NA	11	14	11	14	13	8	9	9	10	9	11	13	7
AR	6.15	7.46	6.63	7728	7.19	5.75	6.49	6.09	6.89	6.97	6.48	7.78	5.81
He	0.592	0.658	0.604	0.604	0.640	0.624	0.665	0.623	0.600	0.495	0.562	0.675	0.603
Ho	0.667	0.733	0.529	0.529	0.691	0.635	0.674	0.739	0.610	0.515	0.565	0.638	0.742
F_{IS}	-0.135	**-0.115**	0.124	-0.014	-0.081	-0.018	-0.015	**-0.188**	-0.017	-0.041	-0.017	0.055	**-0.235**

Table S3 Variable nucleotide positions of the cyt *b* haplotypes observed in *Solea solea* (Part I)

	Nucleotide position																																					
H	1	11	13	19	23	25	31	32	88	91	97	134	136	139	148	155	157	158	161	164	167	176	184	186	193	202	205	208	236	241	247	253	256	259	262	274	301	305	307
H1	T	A	C	G	G	C	A	C	A	A	C	T	A	A	A	C	A	T	C	C	G	G	C	C	C	G	T	A	G	T	T	C	A	A	A	A	T	A	T
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
H8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
H10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
H14	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
H17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
H19	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
H23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
H25	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H26	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
H29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H30	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H31	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H32	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H36	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H37	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H38	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H39	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H40	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H41	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
H42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H44	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H45	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table S3 Variable nucleotide positions of the cyt *b* haplotypes observed in *Solea solea* (Part II)

	Nucleotide position																																						
H	1	11	13	19	23	25	31	32	88	91	97	134	136	139	148	155	157	158	161	164	167	176	184	186	193	202	205	208	236	241	247	253	256	259	262	274	301	305	307
H46	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
H47	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H48	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H49	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H50	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H51	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H52	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H53	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H54	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
H55	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H56	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
H57	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H58	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H59	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H60	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H61	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H62	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
H63	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H64	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H65	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H66	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H67	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H68	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H69	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H70	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H71	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
H72	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H73	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H74	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
H75	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H76	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H77	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H78	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H79	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H80	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table S3 Variable nucleotide positions of the cyt *b* haplotypes observed in *Solea solea* (Part III)

	Nucleotide position																																								
H	1	11	13	19	23	25	31	32	88	91	97	134	136	139	148	155	157	158	161	164	167	176	184	186	193	202	205	208	236	241	247	253	256	259	262	274	301	305	307
H81	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
H82	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H83	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
H84	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H85	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
H86	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H87	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H88	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H89	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H90	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
H91	.	.	.	.	.	.	.	A	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H92	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H93	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
H94	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H95	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H96	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H97	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H98	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H99	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H100	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H101	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H102	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H103	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.
H104	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H105	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H106	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
H107	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table S3 Variable nucleotide positions of the cyt *b* haplotypes observed in *Solea solea* (Part IV)

H	Nucleotide position 316	319	328	331	338	343	349	352	364	365	370	373	379	382	385	391	394	403	409	430	443	445	451	454	457	461	466	469	472	494	505	506	529	532	544	545	547	559	568	587
H1	A	G	G	G	G	C	T	A	A	A	T	T	A	A	G	A	G	A	T	G	G	A	C	A	G	A	A	A	A	G	A	A	T	C	T	A	A	T	A	G
H2	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H3	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H4	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H5	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H6	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H7	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H8	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H11	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H12	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H13	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H14	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
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H17	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H18	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.
H19	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H20	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.
H21	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H22	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H23	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.
H24	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H25	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H26	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H27	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H28	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H29	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A
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H31	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.
H32	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.
H33	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H36	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H37	.	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H38	.	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H39	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H40	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	.
H41	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H42	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H44	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H45	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.

Table S3 Variable nucleotide positions of the cyt *b* haplotypes observed in *Solea solea* (Part V)

	Nucleotide position																																							
H	316	319	328	331	338	343	349	352	364	365	370	373	379	382	385	391	394	403	409	430	443	445	451	454	457	461	466	469	472	494	505	506	529	532	544	545	547	559	568	587
H46	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H47	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H48	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H49	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H50	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H51	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.
H52	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H53	.	A	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H54	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H55	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H56	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H57	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.
H58	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.
H59	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H60	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H61	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.
H62	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H63	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H64	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H65	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H66	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H67	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H68	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H69	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.
H70	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.
H71	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H72	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H73	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H74	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H75	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H76	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H77	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H78	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H79	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H80	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table S3 Variable nucleotide positions of the cyt *b* haplotypes observed in *Solea solea* (Part VI)

	Nucleotide position																																						
H	316	319	328	331	338	343	349	352	364	365	370	373	379	382	385	391	394	403	409	430	443	445	451	454	457	461	466	469	472	494	505	506	529	532	544	545	547	559	568	587
H81	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H82	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H83	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H84	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H85	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H86	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H87	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.
H88	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H89	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.
H90	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H91	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H92	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H93	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H94	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H95	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H96	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H97	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.
H98	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H99	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
H100	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H101	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H102	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H103	.	A	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.
H104	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H105	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	C	.	.	.	.	.
H106	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H107	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.

Table S4 Haplotype distribution of the cyt *b* haplotypes of *Solea solea* – Part I

Pop	TEX07	BEL08S	BEL07S	BEL07F	BEL08J	BEL08F	BISA07	BISB07	BISC07	CEL08	ENG08	GER07	IS08	KATB07	NOR07	NOR08	SKA07	TEX06	THA07	THA07J	THA08	ZAN06	ZAN07	SUM
N	48	45	10	3	27	52	3	5	26	44	48	31	44	39	14	23	32	21	17	17	57	15	30	651
H1	5	1	3		1	7			2	1	6	3	3	3		3	3	2			5	2	2	**52**
H2	21	21	3	1	10	19	2	2	13	18	22	13	20	15	3	10	5	9	6	6	28	5	10	**262**
H3	3	1			2	1		1	1	3	1		2	3	1		2		4	2	2		1	**30**
H4	2	2			1				1	1	1	2		1					1	1	1		1	**15**
H5	1	1			1			1		5			1	2	1	1	3		1		2	1	1	**22**
H6	1																							**1**
H7	3	1				2								1						1				**8**
H8	1	1		1	5	1				5	1	3	1		1	1	2	1		2	3		1	**30**
H9	1																							**1**
H10	1																							**1**
H11	1																							**1**
H12	1																							**1**
H13	2									1						1								**4**
H14	3	2			1	2			1	1	2				1		1	2	3	1	3	2	5	**30**
H15	1																							**1**
H16	1																							**1**
H17		1																						**1**
H18		1									1						1				1	1		**5**
H19		1																						**1**
H20		3	1								1		1	1	1	1		1	1		1			**12**
H21		1																						**1**
H22		2				1						1					2							**6**
H23		1				1			2	2	2					2		1				1		**12**
H24		1				1																		**2**
H25		1				1							1				1			1		1		**6**
H26		1																						**1**
H27		1																						**1**
H28		1											1											**2**
H29			1								1													**2**
H30			1			1					1	1	1			1		1			1			**8**
H31			1																					**1**
H32				1																				**1**
H33					1																			**1**
H34					1																			**1**
H35					1																			**1**
H36					1				1															**2**
H37					1																			**1**
H38					1						1		1											**3**
H39						1						1					1							**3**
H40						1																		**1**

Table S4 Haplotype distribution of the cyt *b* haplotypes of *Solea solea* -Part II

Pop	TEX07	BEL08S	BEL07S	BEL07F	BEL08J	BEL08F	BISA07	BISB07	BISC07	CEL08	ENG08	GER07	IS08	KATB07	NOR07	NOR08	SKA07	TEX06	THA07	THA07J	THA08	ZAN06	ZAN07	SUM
N	48	45	10	3	27	52	3	5	26	44	48	31	44	39	14	23	32	21	17	17	57	15	30	651
H41						1								1					1					**3**
H42						1																		**1**
H43						1							1											**2**
H44						2					1	1			1		1							**6**
H45						1						1												**2**
H46						1																		**1**
H47						1																		**1**
H48						1																		**1**
H49						1				1														**2**
H50						1																		**1**
H51						1																		**1**
H52						1																		**1**
H53							1						1											**2**
H54								1					1										1	**3**
H55									1															**1**
H56									1															**1**
H57									1															**1**
H58									1															**1**
H59									1															**1**
H60										1								1				1		**3**
H61										1														**1**
H62										1	1												1	**3**
H63										1		1												**2**
H64										1			2											**3**
H65										1														**1**
H66											1													**1**
H67											1	1												**2**
H68											2													**2**
H69											2						1			1				**4**
H70												1												**1**
H71												2										1		**3**
H72													1											**1**
H73													1											**1**
H74													1											**1**
H75													1											**1**
H76													1											**1**
H77													1											**1**
H78													1											**1**
H79														6			3							**9**
H80														3	1		3						1	**8**

Table S4 Haplotype distribution of the cyt *b* haplotypes of *Solea solea* -Part III

Pop	TEX07	BEL08S	BEL07S	BEL07F	BEL08J	BEL08F	BISA07	BISB07	BISC07	CEL08	ENG08	GER07	IS08	KATB07	NOR07	NOR08	SKA07	TEX06	THA07	THA07J	THA08	ZAN06	ZAN07	SUM
N	48	45	10	3	27	52	3	5	26	44	48	31	44	39	14	23	32	21	17	17	57	15	30	651
H81														1										**1**
H82														1										**1**
H83														1										**1**
H84															1								1	**2**
H85															1									**1**
H86															1					1				**2**
H87															1									**1**
H88																1				1				**2**
H89																1								**1**
H90																1							1	**2**
H91																	1							**1**
H92																	1							**1**
H93																	1				1			**2**
H94																		1						**1**
H95																		1						**1**
H96																		1			2			**3**
H97																					1			**1**
H98																					1			**1**
H99																					1			**1**
H100																					1			**1**
H101																					1			**1**
H102																					1			**1**
H103																					1			**1**
H104																							1	**1**
H105																							1	**1**
H106																							1	**1**
H107																							1	**1**

Table S5 *Solea solea*. Pairwise F_{ST} (above diagonal) and R_{ST} (below diagonal) estimates of the microsatellite genotypes. Significant values at P <0.05 in bold. Significant after Bonferroni correction (P <0.0001) in bold with asterisk.

	STO07	KATA07	KATB07	SKA07	GER07	LINC07	LINC08	NOR07	NOR08	THA07	THA08	BEL07S	BEL07F	BEL08S	BEL08F
STO07		0.000	0.003	0.001	0.003	0.002	**0.007**	**0.010**	0.005	**0.003**	0.003	**0.006**	**0.005**	**0.009**	**0.004**
KATA07	-0.003		-0.003	0.001	-0.001	0.001	0.002	**0.001***	0.001	**0.003**	-0.002	**0.005**	**0.003**	**0.006**	**0.005**
KATB07	-0.007	-0.005		0.004	-0.001	0.002	0.001	**0.005**	**0.006**	0.002	0.003	0.003	**0.004**	0.004	0.005
SKA07	-0.010	-0.006	-0.009		0.002	**0.005**	0.004	**0.008**	**0.011**	**0.006**	0.005	**0.006**	**0.009**	0.008	**0.006**
GER07	-0.003	-0.008	-0.008	-0.007		0.002	0.002	0.004	0.002	0.003	-0.001	0.005	0.005	0.001	0.001
LINC07	-0.003	-0.002	-0.004	-0.002	-0.004		0.002	**0.003**	0.006	-0.001	-0.001	0.001	-0.001	0.003	-0.001
LINC08	0.006	0.004	0.007	0.008	0.001	-0.005		-0.001	0.006	0.002	0.003	-0.001	**0.002**	0.000	0.000
NOR07	0.008	0.004	0.009	0.009	0.000	0.005	-0.001		0.012	**0.001**	**0.005**	0.001	**0.001**	-0.003	0.000
NOR08	0.002	-0.002	-0.001	0.003	-0.007	-0.005	0.009	0.000		0.006	0.001	**0.009**	**0.007**	0.007	0.006
THA07	0.002	-0.001	0.002	0.003	-0.004	-0.003	-0.004	-0.004	-0.005		0.003	0.000	0.001	0.002	-0.001
THA08	0.009	-0.003	0.009	0.011	0.000	0.003	-0.003	0.001	0.006	-0.002		0.004	0.000	0.003	0.000
BEL07S	0.000	-0.001	0.003	0.002	-0.002	-0.001	0.001	-0.005	-0.003	-0.004	0.000		-0.001	0.002	0.000
BEL07F	0.015	0.014	**0.017**	0.021	0.013	0.001	0.002	0.003	0.002	0.002	**0.008**	0.004		**0.002**	0.000
BEL08S	0.008	0.009	0.010	0.010	0.003	0.001	-0.003	-0.008	0.002	-0.003	0.006	-0.002	0.001		0.000
BEL08F	0.011	0.008	0.007	0.010	0.003	-0.005	-0.002	0.006	0.001	0.001	0.010	0.007	0.002	0.001	
CEL08	-0.001	-0.003	-0.001	0.001	-0.005	-0.002	0.002	-0.001	-0.005	-0.005	0.000	-0.004	0.007	0.001	0.006
IS08	0.006	0.010	0.002	0.003	0.000	0.003	**0.008**	0.007	0.003	0.004	**0.020**	0.009	**0.018**	0.003	0.002
ENG08	-0.003	-0.001	0.001	0.001	-0.001	-0.009	-0.007	0.001	-0.001	-0.005	-0.002	-0.003	0.000	**-0.001**	-0.001
WCH09	-0.001	-0.004	-0.002	0.000	-0.005	-0.005	-0.003	0.006	0.000	-0.003	-0.002	0.001	**0.010**	0.005	0.004
BISA07	0.009	-0.001	0.007	0.009	-0.002	0.001	0.002	0.000	-0.005	-0.004	-0.004	-0.001	0.007	0.003	**0.007**
BISB07	0.014	0.006	0.012	0.016	0.002	-0.001	0.001	-0.003	-0.010	-0.004	0.001	0.000	-0.003	-0.001	-0.001
BISC07	0.010	0.010	0.006	0.010	0.003	-0.005	-0.004	0.006	0.003	0.000	0.011	0.007	0.002	-0.001	-0.009
TEX06	0.001	-0.002	0.004	0.005	0.007	0.004	0.007	0.013	0.015	0.006	0.006	0.003	**0.017**	0.013	**0.018**
TEX07	**0.032**	0.016	**0.037**	**0.0422***	**0.029**	0.025	**0.021**	**0.020**	**0.023**	**0.018**	0.004	**0.015**	**0.018**	0.027	**0.039**
THA07J	0.001	0.002	0.002	0.004	0.000	-0.011	-0.003	0.002	-0.007	-0.005	0.006	-0.002	-0.004	-0.003	-0.007
ZAN06	0.000	0.001	0.008	0.007	**0.009**	0.000	0.007	0.014	0.010	0.006	0.003	0.002	**0.013**	0.015	**0.019**
ZAN07	0.002	0.005	0.000	0.002	0.000	-0.005	-0.004	0.002	0.005	-0.001	0.010	0.002	0.005	-0.002	-0.004
BEL08J	**0.035**	**0.033**	**0.026**	**0.034**	**0.023**	0.013	0.008	**0.025**	0.026	**0.019**	**0.034**	**0.0317***	**0.022**	0.013	0.002

Table S5 cont.

	CEL08	IS08	ENG08	WCH09	BISA07	BISB07	BISC07	TEX06	TEX07	THA07J	ZAN06	ZAN07	BEL08J
STO07	**0.004**	**0.007**	0.002	**0.009**	**0.007**	**0.011**	**0.009**	0.008	0.003	0.004	0.004	0.003	0.009
KATA07	**0.002**	**0.007**	0.002	**0.007**	**0.006**	**0.009**	**0.008**	0.004	0.001	-0.002	0.001	0.003	0.007
KATB07	**0.003**	0.003	0.001	**0.005**	**0.006**	**0.008**	0.004	0.003	0.004	-0.001	0.000	0.002	0.006
SKA07	**0.005**	**0.008**	0.005	**0.009**	**0.009**	**0.008**	**0.011**	**0.009**	**0.003**	0.003	0.002	**0.004**	0.012
GER07	**0.003**	0.004	0.002	0.004	0.004	0.005	0.006	0.004	0.003	0.001	-0.001	0.002	0.004
LINC07	**0.003**	**0.004**	-0.003	0.001	-0.001	0.004	0.003	0.001	0.002	0.003	0.002	-0.001	0.008
LINC08	**0.000**	-0.001	0.001	0.002	0.001	0.001	-0.002	0.000	-0.001	0.001	-0.001	0.000	0.007
NOR07	**0.004**	**0.002**	0.002	**0.001**	**0.004**	0.005	**0.005**	**0.002**	**0.000**	0.002	0.002	0.000	0.003
NOR08	0.003	0.007	0.004	0.009	0.008	0.007	**0.012**	**0.012**	0.003	0.003	0.003	**0.012**	**0.015**
THA07	0.001	0.000	-0.002	0.001	**0.001**	0.004	0.001	0.001	**0.003**	0.003	0.002	-0.001	**0.005**
THA08	0.001	0.005	0.001	0.001	0.002	0.003	0.006	0.002	0.001	-0.001	0.002	0.002	0.007
BEL07S	0.002	0.001	0.000	0.003	**0.003**	0.002	**0.002**	0.001	0.001	0.001	**0.002**	-0.003	0.003
BEL07F	**0.003**	**0.004**	**0.000**	0.002	**0.004***	**0.005**	0.005	0.001	0.001	-0.002	**0.002**	-0.001	0.004
BEL08S	**0.005**	0.002	0.002	0.004	0.003	0.002	**0.006**	0.003	0.003	0.002	0.004	0.001	0.006
BEL08F	**0.000**	0.000	-0.003	-0.002	-0.001	0.000	0.000	-0.001	**0.002**	0.001	0.002	-0.002	0.002
CEL08		0.001	0.000	0.002	**0.002**	0.001	**0.001**	0.004	**0.000**	0.000	**0.002**	**0.003**	0.006
IS08	0.004		0.003	0.001	0.001	0.002	-0.001	0.003	**0.003**	0.003	**0.003**	-0.001	0.005
ENG08	-0.002	0.007		0.000	0.000	0.002	0.000	0.001	**0.005**	0.000	0.002	-0.001	0.004
WCH09	-0.003	**0.007**	-0.005		-0.001	0.002	0.001	0.001	0.003	0.001	0.002	0.002	0.003
BISA07	-0.003	**0.012**	0.000	-0.002		-0.001	0.000	0.002	**0.006**	0.005	0.004	**0.004**	**0.006**
BISB07	0.001	**0.011**	0.000	0.004	-0.004		-0.001	0.006	0.006	0.005	**0.006**	0.005	**0.006**
BISC07	0.005	-0.001	-0.002	0.002	**0.007**	0.001		0.002	**0.005**	0.006	**0.004**	0.002	0.007
TEX06	0.004	**0.022**	0.003	0.004	**0.012**	**0.020**	**0.018**		0.003	0.004	-0.001	0.000	0.008
TEX07	**0.020**	**0.059**	0.018	**0.020**	0.013	**0.019**	**0.041**	0.014		0.000	0.000	0.001	0.007
THA07J	-0.002	0.001	-0.008	-0.001	0.002	-0.002	-0.007	0.004	**0.025**		0.000	0.001	0.001
ZAN06	0.005	**0.027***	-0.002	0.003	0.008	**0.015**	**0.020**	-0.003	0.009	0.004		0.002	**0.008**
ZAN07	0.002	-0.001	-0.002	0.002	0.008	0.005	-0.006	0.007	0.039	-0.007	**0.013**		0.001
BEL08J	**0.026**	0.010	0.019	**0.022**	**0.031**	**0.024**	-0.002	**0.037**	**0.0708***	0.005	**0.0514***	0.005	

Table S6 *Solea solea*. Pairwise D_{est} values of the microsatellite genotypes (Jost 2008)

	STO07	KATA07	KATB07	SKA07	GER07	LINC07	LINC08	NOR07	NOR08	THA07	THA08	BEL07s	BEL07f	BEL08s	BEL08f	CEL08	IS08	ENG08	WCH09	BISA07	BISB07	BISC07	TEX06	TEX07	THA07j	ZAN06	ZAN07	BEL08j
STO07	--	0.000	0.000	0.000	0.001	0.000	0.004	0.011	0.001	0.003	0.001	0.003	0.003	0.015	0.007	0.004	0.010	0.003	0.009	0.006	0.012	0.010	0.007	0.002	0.002	0.003	0.002	0.004
KATA07		--	0.000	0.000	0.000	0.000	0.003	0.020	0.000	0.003	0.000	0.008	0.004	0.009	0.009	0.001	0.014	0.001	0.012	0.007	0.011	0.012	0.004	0.000	0.000	0.002	0.002	0.004
KATB07			--	0.000	0.000	0.000	0.000	0.004	0.002	0.001	0.001	0.003	0.002	0.006	0.006	0.001	0.004	0.000	0.003	0.005	0.005	0.002	0.003	0.002	0.000	0.000	0.000	0.003
SKA07				--	0.000	0.004	0.002	0.009	0.006	0.006	0.003	0.006	0.012	0.010	0.004	0.002	0.005	0.003	0.008	0.005	0.003	0.006	0.007	0.002	0.002	0.000	0.006	0.005
GER07					--	0.000	0.001	0.002	0.001	0.001	0.000	0.005	0.008	0.002	0.000	0.001	0.002	0.000	0.003	0.003	0.004	0.004	0.002	0.002	0.002	0.000	0.000	0.001
LINC07						--	0.000	0.000	0.002	0.000	-0.001	0.001	0.000	0.002	0.000	0.004	0.006	-0.001	0.001	0.000	0.003	0.001	0.000	0.001	0.002	0.002	0.000	0.008
LINC08							--	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003
NOR07								--	0.008	0.000	0.006	0.001	0.002	-0.001	0.000	0.004	0.001	0.000	0.000	0.004	0.011	0.004	0.002	0.000	0.001	0.001	0.000	0.002
NOR08									--	0.001	0.000	0.003	0.007	0.007	0.001	0.000	0.001	0.000	0.004	0.002	0.003	0.007	0.011	0.000	0.004	0.001	0.007	0.013
THA07										--	0.002	0.001	0.002	0.002	0.000	0.000	0.000	-0.002	0.000	0.001	0.003	0.000	0.001	0.002	0.000	0.001	0.000	0.003
THA08											--	0.004	0.000	0.003	0.000	0.001	0.008	0.000	0.003	0.001	0.002	0.004	0.000	0.000	0.000	0.002	0.000	0.009
BEL07s												--	0.000	0.003	0.000	0.002	0.001	0.001	0.001	0.004	0.001	0.002	0.001	0.002	0.000	0.003	-0.001	0.001
BEL07f													--	0.000	0.000	0.003	0.007	0.000	0.001	0.005	0.003	0.003	0.000	0.001	0.000	0.002	0.000	0.002
BEL08s														--	0.000	0.005	0.003	0.001	0.004	0.004	0.004	0.007	0.006	0.003	0.002	0.007	0.001	0.005
BEL08f															--	0.000	0.000	-0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000
CEL08																--	0.000	0.000	0.001	0.001	0.000	0.000	0.003	0.000	0.000	0.000	0.002	0.001
IS08																	--	0.003	0.000	0.002	0.001	0.000	0.001	0.003	0.002	0.002	0.000	0.003
ENG08																		--	0.000	0.000	0.002	0.000	0.001	0.003	0.000	0.002	0.000	0.001
WCH09																			--	0.000	0.000	0.000	0.000	0.005	0.001	0.000	0.002	0.001
BISA07																				--	0.000	0.000	0.000	0.010	0.002	0.001	0.004	0.004
BISB07																					--	0.000	0.005	0.004	0.003	0.003	0.005	0.003
BISC07																						--	0.001	0.008	0.003	0.002	0.001	0.002
TEX06																							--	0.004	0.001	0.000	0.000	0.008
TEX07																								--	0.000	0.000	0.000	0.014
THA07j																									--	0.000	0.000	0.000
ZAN06																										--	0.002	0.009
ZAN07																											--	0.000
BEL08j																												--

Table S7 Pairwise F_{ST} based on mitochondrial data of *Solea solea*. Significant values (α = 0.05) in bold. Significant after Bonferroni correction (α =0.0001) in bold with asterisk

	SKA07	KATB07	GER07	NOR07	NOR08	THA07	THA08	BEL07s	BEL07f	BEL08s	BEL08f	CEL08	IS08	ENG08	BISA07	BISB07	BISC07	TEX06	TEX07	THA07j	ZAN06	ZAN07	BEL08j
SKA07	-	-0.015	**0.042**	-0.022	0.009	0.031	**0.037**	0.007	-0.083	**0.048**	0.005	**0.086***	0.028	0.015	-0.035	-0.033	**0.043**	0.020	**0.039**	0.033	0.002	0.023	0.032
KATB07		-	**0.040**	-0.010	0.022	0.037	**0.043**	0.033	-0.103	**0.049**	0.011	**0.090***	**0.036**	0.025	-0.053	-0.010	**0.045**	0.031	**0.039**	0.036	0.024	0.029	**0.045**
GER07			-	-0.011	0.006	0.027	-0.012	**0.092**	0.037	-0.004	-0.002	0.021	-0.008	-0.004	-0.021	-0.028	-0.009	-0.013	-0.012	-0.012	-0.001	-0.005	0.003
NOR07				-	-0.021	-0.020	-0.016	0.014	-0.070	-0.011	-0.025	0.018	-0.022	-0.017	-0.086	-0.088	-0.016	-0.030	-0.012	-0.020	-0.037	-0.028	-0.023
NOR08					-	0.035	0.000	-0.011	0.007	0.026	-0.006	**0.053**	0.001	-0.021	0.008	-0.028	-0.001	-0.019	0.001	0.017	-0.022	0.006	0.015
THA07						-	0.010	**0.137**	0.063	-0.008	0.011	0.017	0.014	0.025	0.033	-0.050	-0.004	-0.006	0.024	-0.013	-0.014	-0.007	-0.002
THA08							-	**0.089**	0.044	0.001	-0.003	0.011	-0.011	-0.005	0.016	-0.038	-0.011	-0.024	-0.011	-0.014	-0.018	-0.011	-0.011
BEL07s								-	0.002	**0.130**	0.041	**0.212**	0.076	0.017	0.060	0.058	**0.110**	0.049	0.083	**0.129**	0.038	0.080	**0.110**
BEL07f									-	0.019	-0.036	0.129	0.023	0.004	-0.219	-0.029	0.050	0.018	0.031	0.027	-0.015	-0.006	0.043
BEL08s										-	0.012	0.001	0.003	0.021	-0.043	-0.059	-0.015	-0.003	0.010	-0.019	-0.006	0.002	0.002
BEL08f											-	**0.031**	-0.004	-0.008	-0.042	-0.034	0.005	-0.014	-0.007	-0.011	-0.018	-0.010	-0.004
CEL08												-	0.010	**0.043**	0.068	-0.037	-0.005	0.011	0.020	-0.010	0.004	0.016	-0.004
IS08													-	-0.002	-0.019	-0.064	-0.004	-0.016	-0.008	-0.009	-0.015	-0.005	-0.011
ENG08														-	-0.008	-0.022	0.003	-0.019	-0.005	0.003	-0.018	-0.003	0.005
BISA07															-	-0.077	-0.006	0.011	0.003	-0.005	-0.010	-0.019	0.028
BISB07																-	-0.052	-0.035	-0.023	-0.033	-0.055	-0.042	-0.035
BISC07																	-	-0.017	-0.003	-0.015	-0.023	-0.007	-0.005
TEX06																		-	-0.017	-0.023	-0.039	-0.023	-0.023
TEX07																			-	-0.013	-0.010	-0.013	-0.006
THA07j																				-	-0.023	-0.025	-0.023
ZAN06																					-	-0.025	-0.030
ZAN07																						-	-0.017
BEL08j																							-

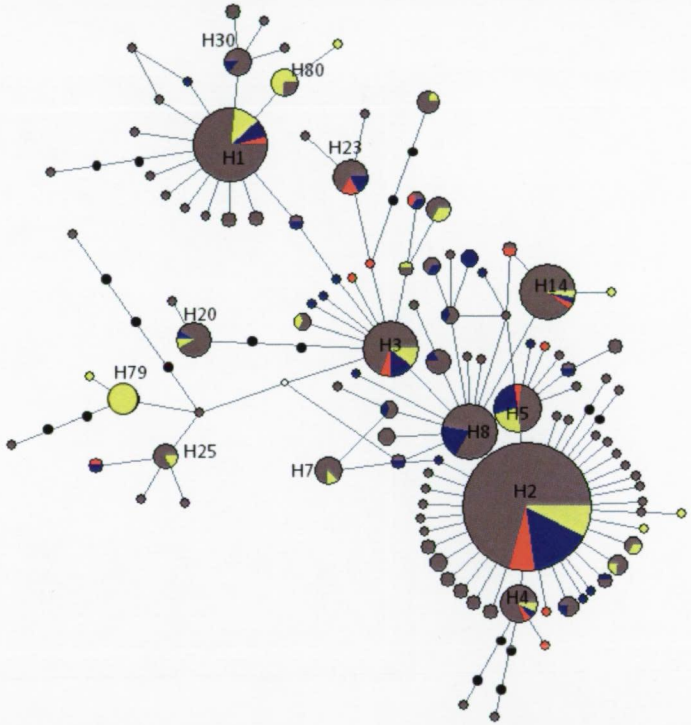
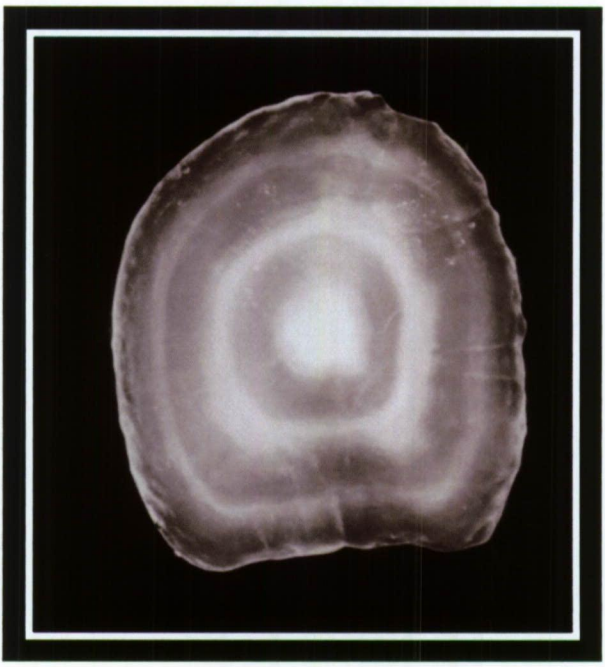


Fig. S1 *Solea solea*. Median Joining Network of haplotypes found in this study (yellow= 'Skagerrak/Kattegat'; grey='North Sea'; blue= 'Irish Sea/Celtic Sea', red='Biscay'). The most common haplotypes are assigned by their name (see Table S3 and Table S4).



Chapter 2

Microchemical variation in juvenile sole (*Solea solea*) otoliths as a powerful tool for studying connectivity in the North Sea

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ABSTRACT

Estimating connectivity between juvenile and adult habitats can provide an important contribution to effective fisheries management, through the better understanding of population resilience to harvesting pressure. Indirect methods for quantifying connectivity, such as geochemical or genetic techniques, allow us to assign adults from various sampling regions to their natal location, provided that natal origin data can be defined. The elemental composition of otoliths from juvenile sole (*Solea solea*) collected at four sampling locations in the Southern Bight of the North Sea was measured using laser-ablation inductively-coupled plasma mass spectrometry, in order to determine elemental fingerprints indicative of distinct nursery grounds. Significant differences in elemental composition were detected among the four locations, with Na, Sr, Ba, Mn and Rb concentrations varying the most between groups. A discriminant model resulted in high assignment proportions of the juvenile fish to their respective nursery grounds with a total jackknife reclassification success of 88 %. Even though some interannual variability in otolith chemistry was observed in juveniles from the Scheldt estuary, spatial patterns seemed to dominate. Our results constitute a firm basis for future investigations on nursery area contributions and quality, adult dispersal history and applications of population traceability.

Published in Marine Ecology Progress Series (2010), 401, 211-220

INTRODUCTION

Understanding population connectivity is an important component of effective fisheries management, because the exchange of individuals among geographically separate groups determines the colonization rate of new habitats, the resilience to harvesting and the success of management measures such as marine protected areas (Thorrold et al. 2001). Furthermore, essential habitats must be identified based on knowledge about the spatial scales of movement of individuals and habitat use of different life history stages in order to select habitats for conservation (Beck et al. 2001; Fogarty & Botsford 2007).

Connectivity in marine fish populations can be estimated from a variety of direct and indirect sources. Direct physical tagging is widely applied and these studies can provide insights about movement patterns and habitat range. Nevertheless, most tagging studies focus on adult fish due to the limited feasibility of physical tags in small or juvenile fish (Palumbi 2004b; Cowen & Sponaugle 2009) and may require the recapture of the fish, which poses many challenges in an open environment such as the ocean (Thorrold & et al. 2002). Indirect methods using genetic markers can also be used to investigate connectivity between populations by calculating the amount of gene flow and the extent of genetic differentiation. Furthermore, genetic methods provide insights on the spatio-temporal connectivity on an evolutionary time scale (in the order of hundreds of generations) (Hedgecock et al. 2007). In marine fish, however, the chances for detecting strong genetic structure, applicable into management decisions, are low because even few migrants suffice to prevent detectable differentiation (Hellberg et al. 2002; Hartl & Clark 2007). Since the 1980's, otolith elemental composition has increasingly been used to describe life histories of fish, study small scale variability in seasonal and within generation migrations, and identify the various environments fish have experienced. The use of trace elements in otoliths is based on the empirical evidence that fish incorporate elements from their environments and that these elements are permanently deposited in their continuously growing otoliths (Campana 1999; Thresher 1999). Consequently, the chemical composition of otoliths differs among fish from different geographical areas and constitutes a powerful multi-elemental signal reflecting the surrounding physical and chemical environment (Vasconcelos 2007). Juvenile fish that have

lived in different coastal environments or estuaries are often characterized by a distinct otolith composition, referred to as the elemental fingerprint. Analysis of otolith composition has been successfully applied in a wide range of flatfish species living in different habitats (de Pontual et al. 2000; Forrester & Swearer 2002; Gillanders 2002a; Geffen et al. 2003; Brown 2006; Vasconcelos 2007). Because different nursery areas can be defined by a distinct otolith element signature derived from the composition of juveniles sampled there, adult fish can be assigned to their nursery area by comparing the elemental composition of the central otolith zone, which represents the first year of life, with the specific nursery signature (Thorrold et al. 2001; Brown 2006; Vasconcelos et al. 2008). Results can provide crucial information on migration distance, mixed-stock analyses of feeding aggregations and the relative importance of different nurseries, estimated as the fraction of successful recruits originating from a specific nursery area (Gillanders 2002a).

Sole (*Solea solea;* Soleidae; Teleostei) is a commercially important flatfish in the Northeastern Atlantic and to a lesser extent in the Mediterranean Sea, but is overexploited throughout most of its range. In the North Sea, sole spawn from early April until August, with a main peak in May, at water temperatures ranging between 8 and 12°C (Van der Land 1991; Rijnsdorp et al. 1992). There are five main spawning grounds in the North Sea, namely the inner German Bight, in the eastern English Channel, off the Belgian Coast, in the Thames and on the Norfolk Banks (Russell 1976; Rijnsdorp et al. 1992). About 30 days after hatching, the pelagic larvae metamorphose and settle in shallow marine coastal habitats and estuaries that provide suitable feeding conditions (van der Veer et al. 2001; Amara 2004). The juvenile sole stay in these nursery areas for up to two years before they move offshore and reach maturity from the age of three onwards (Pawson 1995; Burt & Millner 2008). Sole nurseries are situated along the shallow muddy and sandy coasts of the North Sea and in the estuaries. Because estuaries and coastal areas are characterized by a distinct water chemistry compared to the open sea, and because spawning areas are not far from the nursery grounds in the North Sea, the likelihood for successful population assignments using otolith microchemistry is expected to be high for sole, as has been shown elsewhere (Bay of Biscay (de Pontual et al. 2000) – Portuguese coast (Vasconcelos 2007; Vasconcelos et al. 2008) – Thames (Leakey et al. 2009)).

Tagging experiments on sole in the Southern North Sea and the Irish Sea (Symonds & Rogers 1995; Burt & Millner 2008) indicate that sole undertake relatively short migrations away from the coastal nursery and spawning grounds. Movements are seasonal with mature sole migrating inshore to spawn in spring and moving offshore in winter. In the North Sea mainly northward offshore movements have been observed, which may be associated with the northward residual current. Few sole migrate from the Belgian and Dutch coast into the Thames area, possibly pointing to a stable separation between populations. Within the eastern English Channel strong site fidelity has been observed (Burt & Millner 2008).

The entire sole stock in the North Sea region (ICES area IV) is currently managed as a single unit, potentially leading to over-harvesting of unrecognized populations. If the current management unit consists of several subunits, fishing pressure forms a threat to the maintenance of intraspecific (phenotypic and genetic) diversity at the species and population level (Frank & Brickman 2001; Secor et al. 2009). A thorough and integrated reassessment of the spatial and temporal scale of population connectivity using up to date methodologies is urgently needed to avoid the extirpation of small populations. Although various studies have analysed the microchemical fingerprint signal in sole, all studies focused on a relatively small area, while almost nothing is known about the connectivity of sole at the North Sea scale, between local populations across the English Channel or about the relative contributions of the neighbouring nursery grounds.

The main objectives of the present study are (1) to characterise juvenile otolith microchemical fingerprints from four known nursery areas for sole in the North Sea (along coast and on opposite sides of the Southern North Sea) and (2) to assess the discriminative power of sole microchemistry signatures of nurseries located in the Southern Bight of the North Sea. Hence, we aim at testing the applicability of otolith microchemistry for tracing back the nursery grounds of origin of adult soles at a larger geographical scale, such as in the dynamic North Sea basin.

MATERIALS AND METHODS

Sampling

Juvenile fish (0-group and 1-group) were collected at four sampling sites in the autumn of 2006 or 2007: in front of the Thames mouth (THA07), off the Humber estuary (Lincolnshire area- LINC07), in the Wadden Sea (near Texel- TEX06) and in the Scheldt estuary (near Zandvliet- ZAN06 and ZAN07) (Fig. 1). We selected these four sampling locations because they are adjacent to known spawning grounds in the Thames, Norfolk Banks, German Bight and Belgian Coast and represent some of the most important nursery areas with high densities of juvenile flatfish (Rijnsdorp et al. 1992; Hamerlynck et al. 1993; Rogers et al. 1998; Beyst et al. 1999; Hostens 2000; van der Veer et al. 2001; Vinagre et al. 2008a).

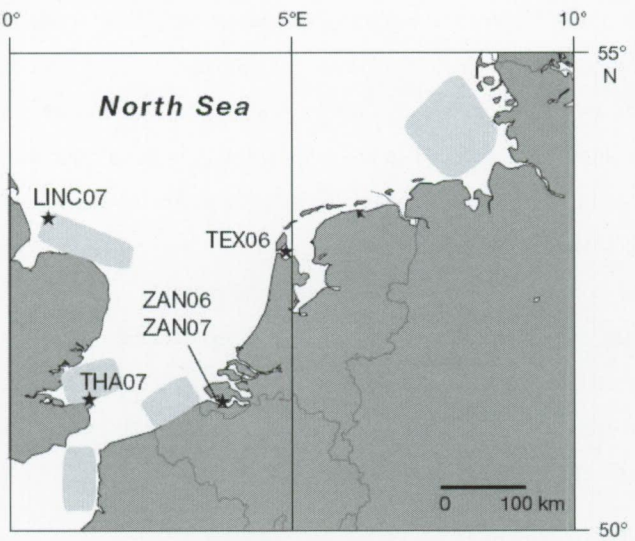


Fig.1 *Solea solea*. Study area showing sampling sites: Lincolnshire near Humber estuary (LINC07), Thames (THA07), Wadden Sea near Texel (TEX06) and Scheldt near Zandvliet (ZAN06/ZAN07). Numbers in the location code indicate sampling year and the shaded areas indicate spawning grounds.

Samples were obtained from standard surveys using a beam trawl, except for the Scheldt estuary, where samples were collected in both 2006 and 2007 with fyke nets placed at an intertidal mud flat (Table 1). The geographical distance between the sampling sites ranged from 193 to 293 km. Abiotic variables were obtained from databases of the Flemish Environment Agency (VMM), from the Dutch Ministry of Transport, Public Works and Water

Management (www.waterbase.nl) or were measured during the surveys. Fish were stored immediately at -20°C and further measurements (total length, weight) and otolith extractions (sagittae) were performed in the laboratory. From each location, the otoliths of 20 to 40 fish less than 20 cm (0-1 group) were selected for otolith microchemistry analysis (Table 1).

Otolith preparation

The left sagitta was used for analysis, except when only the right sagitta could be retrieved. To remove any surface contamination, the otoliths were cleaned using a standard decontamination protocol for otoliths (Otolith Research Laboratory, Bedford Institute of Oceanography). Only analytical grade chemicals and equipment made of teflon, polyethylene or polypropylene were used. Otoliths were cleaned from any adhering tissues, placed in labelled vials, covered with ultrapure water and sonified for five minutes. Otoliths were scrubbed with a nylon brush, triple rinsed with ultrapure water, placed back in the vials and sonified for another three minutes. After cleaning, the otoliths were dried within the vial. The dry otoliths were weighed to the nearest 0.005 mg and then stored in fresh acid washed vials awaiting further preparation. Up to 30 juvenile sole otoliths were mounted on a single geological slide in epoxy resin, lined up with their proximal side facing up. They were ground by hand using a series of grinding papers and polished with diamond paste (3 – 1 - 0.25 µm) until the edge material could be sampled. Specimens from different locations were randomized on each slide (to avoid preparation artefacts). Finally, to remove any remaining surface contamination, each slide was sonified for 10 min in milliQ water and dried under a laminar flow cabinet.

ICP-MS measurement

The chemical composition of the nursery ground portion of the juvenile sole otoliths was determined by laser-ablation inductively-coupled plasma mass spectrometry (LA-ICPMS) analyses using a Finnigan ELEMENT 2 ICP-MS (Thermo Electron Corporation, Bremen) coupled to a UP266 MACRO laser system (New Wave Research). Laser conditions (10 Hz repetition rate, 50 s dwell time, medium resolution) induced a crater of 60 µm diameter at the outermost part of the otolith. Measurements were performed on the otolith edge to ensure that the analyzed material was laid down just before capturing the fish, at the nursery ground. For all the otoliths, the following isotopes were measured: ^{7}Li, ^{23}Na, ^{24}Mg,

^{25}Mg, ^{43}Ca, ^{55}Mn, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{85}Rb, ^{86}Sr, ^{88}Sr, ^{137}Ba, ^{138}Ba and ^{208}Pb. For the elements Mg, Sr and Ba, two isotopes were initially quantified to test for possible mass interference. The isotope with least interference was selected for statistical analyses: ^{24}Mg, ^{86}Sr and ^{138}Ba. Two reference materials (BCR-2 carbonate powder and NIES CRM No.22 Fish Otolith powder (National Institute for Environmental Studies, Japan)) were pressed into pellets for laser ablation. These were included as reference standards with NIST610 glass as calibration standards and were measured between every 15 spots for calibrations. Data below the limits of detection (LOD) were set to the limit of detection. Estimates of precision (% RSD) based on the repeated analyses of the NIST610 standard were determined for each element: ^{7}Li = 3.45 %, ^{23}Na = 2.37 %, ^{24}Mg = 3.02 %, ^{55}Mn = 1.68 %, ^{59}Co = 2.17 %, ^{63}Cu = 2.70 %, ^{66}Zn = 6.61 %, ^{85}Rb = 2.65 %, ^{86}Sr = 3.03 %, ^{138}Ba = 3.64 % and ^{208}Pb= 3.85 %.

Table 1. *Solea solea*. Overview of sampling sites, sampling code, latitude and longitude coordinates of sampling site, sampling year, water temperature (T), salinity (Sal), number of samples analyzed (N), total fish length (TL) (cm) mean ± standard deviation, otolith weight (OW) (mg) mean + standard deviation. *Data not collected but estimated from monthly means (Source 'Waterbase', www.waterbase.nl).

Sampling site	Sampling code	Coordinates	Year	T	Sal	N	TL (cm)	OW (mg)
Zandvliet (Scheldt estuary)	ZAN06	51°24'1"N 4°11'40"E	2006	18.6	11	36	9.9 ± 0.59	1.58 ± 0.20
Zandvliet (Scheldt estuary)	ZAN07	51°24'1"N 4°11'40"E	2007	16	10	30	11.2 ± 0.85	2.17 ± 0.35
Thames	THA07	51°25'14"N 1°23'49"E	2007	17.7	34.2	35	10.1 ± 1.19	1.55 ± 0.29
Texel (Wadden sea)	TEX06	52°57'46"N 4°57'12"E	2006	18*	28.7*	28	9.8 ± 0.90	1.16 ± 0.19
Lincolnshire (Humber estuary)	LINC07	53°19'96"N 0°25'63"E	2007	16.4	32.2	23	17.2 ± 2.29	5.99 ± 1.83

Data analysis

Counts-per-second were processed using the signal integration software GLITTER (GEMOC, Macquarie University) with NIST610 selected as a standard and ^{43}Ca as internal standard. Values were expressed as element concentrations ($\mu g \cdot g^{-1}$), normalized to ^{43}Ca (Longerich et al. 1996). The minimum detection limit at the 99 % confidence level was calculated by the

GLITTER software using an algorithm developed by Longerich et al. (1996) (LOD = 2.3 * √2B with B representing the total number of counts in the background interval) (Van Achterberg et al. 2001). Otolith element concentrations were log_{10}-transformed in order to achieve a normal data distribution and the homogeneity of variances between groups. Since many aspects of growth can influence elemental incorporation, variation in log_{10}-transformed total fish length between sampling sites was examined using a one-way ANOVA. Although total fish length differed significantly between groups (ANOVA, $F_{4,140} = 155.1$; $p < 0.0001$), with mainly the sample LINC07 consisting of larger fish (Table 1), no significant effect was visible of the covariate length on variation in otolith composition (ANCOVA: $F_{10;130} = 1.3$; $p = 0.23$). Variation in otolith composition within and between sampling sites was thus evaluated and quantified using a multiple analysis of variance (MANOVA). *Post-hoc* comparisons using Tukey HSD tests were subsequently applied, to pinpoint the significantly different nurseries.

Forward stepwise Linear Discriminant Function Analysis (LDFA) was used to discriminate between nurseries; including only elements in the model that contribute most to the discrimination. The functions generated were then used to classify fish according to nursery ground. A cross-validation algorithm using a jackknife technique was applied to determine the classification accuracy. A randomization technique was used to test the significance of the observed reclassification success compared to that expected by chance (White & Ruttenberg 2007). Initially, all samples (THA07, LINC07, TEX06, ZAN06 and ZAN07) were used to build the discriminant functions and the jackknife reclassification success was evaluated. In a second analysis, four geographically separated samples THA07, LINC07, TEX06 and ZAN06 were used for the calculation of the discriminant functions, while the omitted ZAN07 sample was used as a test group. The latter model made it possible to assess the influence of annual variation in otolith fingerprints on the spatial discrimination success of juvenile soles. Additionally, inter-annual variation in each element was tested using the consecutive samples from Zandvliet (2006 and 2007) with a pairwise t-test for independent samples. Statistical analyses were performed with the software STATISTICA 8.0 (StatSoft, 2008) and SAS v.9.1 (SAS Institute Inc.); significance level used for all tests was $\alpha < 0.05$.

RESULTS

Ten elements (Na, Mg, Mn, Co, Cu, Zn, Rb, Sr, Ba, Pb) were regularly detected at all sites and thus included in the statistical analyses. For most elements, the mean percentage of data below the detection limit was lower than 10 %, except for the elements Li (30 %), Cu (11 %) and Pb (25 %). For the element Li the percentage of data below the LOD was especially high (60 %) for the samples from LINC07 compared to the other locations. Li was therefore ignored in the statistical analyses. The most abundant trace elements (concentration < 100 $\mu g.g^{-1}$) were Mg (49 $\mu g.\ g^{-1}$), Mn (14 $\mu g.\ g^{-1}$) and Ba (3 $\mu g.\ g^{-1}$) (Table 2).

Table 2. *Solea solea*. Mean ± SD elemental concentrations of otolith edges in $\mu g \cdot g^{-1}$, mean limits of detection (LOD) and percentage of data below the limits of detection (% < LOD) for all elements.

Element	Concentration			LOD	% < LOD
Li	0.9	±	1.36	0.068	30
Na	3103.2	±	361.50	0.107	0
Mg	48.99	±	9.74	0.016	0
Mn	14.1	±	9.62	0.011	0
Co	0.8	±	0.65	0.016	9
Cu	0.3	±	0.22	0.006	11
Zn	0.6	±	0.53	0.014	10
Rb	0.1	±	0.11	0.001	3
Sr	1627.6	±	328.19	0.041	0
Ba	2.9	±	2.31	0.001	0
Pb	0.03	±	0.05	0.001	25

Multi-elemental fingerprints differed significantly among sampling locations (MANOVA: $F_{40;498}$= 30.1; $p < 0.0001$). ANOVA's revealed significant differences in elemental concentration between the sampling sites for 8 of the 10 elements analyzed (Table 3). Post-hoc Tukey results indicated that in most cases, four to six of the elements differed significantly between two locations. Ba, Na and Mg differed among several sampling locations (Fig. 2). The Ba concentration was lower in fish from the English coast compared to the samples from the Wadden Sea and Scheldt estuary (Fig. 2). High Mn concentrations were measured in the samples from Texel, which were also characterized by high Mg concentrations. Samples from Zandvliet 2006 showed high concentrations of Cu, Rb and Ba. Sr concentration was lowest in Zandvliet (1256 $\mu g.g^{-1}$) and highest in the samples from Lincolnshire (2098 $\mu g.g^{-1}$).

Table 3. *Solea solea*. Results of univariate ANOVA comparing the elemental concentration of otoliths between sampling locations. Significant p-values are in **bold** text; df = 4; 140

Element	Adjusted R^2	*F*	p
Na	0.384	23.403	**< 0.0001**
Mg	0.613	57.961	**< 0.0001**
Mn	0.785	132.126	**< 0.0001**
Co	0.024	1.874	0.118
Cu	0.045	2.712	**0.032**
Zn	0.014	1.529	0.197
Rb	0.372	22.329	**< 0.0001**
Sr	0.664	72.154	**< 0.0001**
Ba	0.752	110.225	**< 0.0001**
Pb	0.079	4.075	**0.004**

Among the ten elements included in the stepwise analysis, six elements were entered into the model (Mn, Ba, Sr, Rb, Na and Mg) with Mn, Ba and Sr as the most important. Overall jackknife reclassification success was 88 %, which was significantly higher than the 20 % expected by chance ($p < 0.0001$). The highest classification accuracy (100 %) was obtained for the individuals from THA07, while most classification errors were associated with the samples of ZAN06 (Fig. 3). Seven fish from ZAN06 were assigned to the correct location but to a different year (2007), two were assigned to THA07 and one to LINC07.

A canonical analysis identified three discrete groups of fish differing significantly in their fingerprint pattern: the first group mainly contained the samples from Lincolnshire and Thames; the second group consisted of the samples from Zandvliet (both years) and the third group contained all samples from Texel (Fig. 4). The first discriminant function, which was mainly determined by Ba, Mn and Mg (in growing order of importance), separated these three groups and to some extent also the samples from the Thames from those of Lincolnshire. The second discriminant function was mainly determined by Mn and Sr and to a lesser extent by Mg. Root 1 and 2 explained together 85 % of the variation.

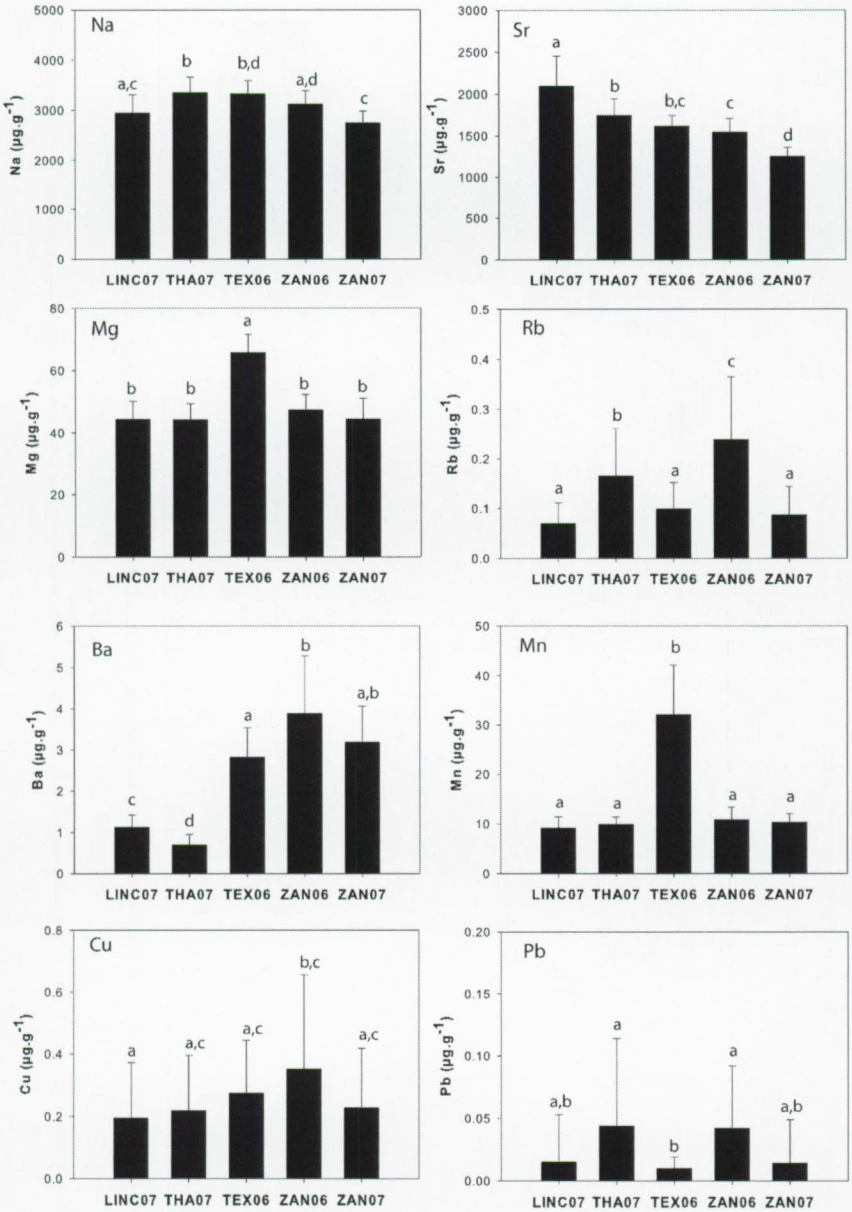


Fig.2 *Solea solea*. Mean + SD elemental concentrations of the 8 elements in otolith edge that differed significantly among the sampling locations (see Table 1 for sample codes). Bars labelled with different lowercase letters are statistically different from each other (*post-hoc* multiple comparison with Tukey HSD test)

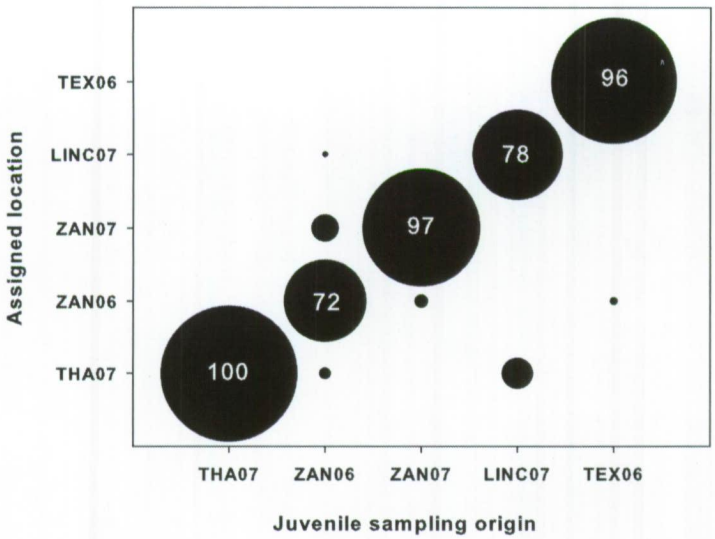


Fig.3 *Solea solea* Allocation of juvenile fish to the sampling groups based on linear discriminant function analysis using the elements Na, Mg, Mn, Rb, Sr and Ba. Circle size is scaled relative to the jackknife reclassification success, which is shown (%) within the larger circles.

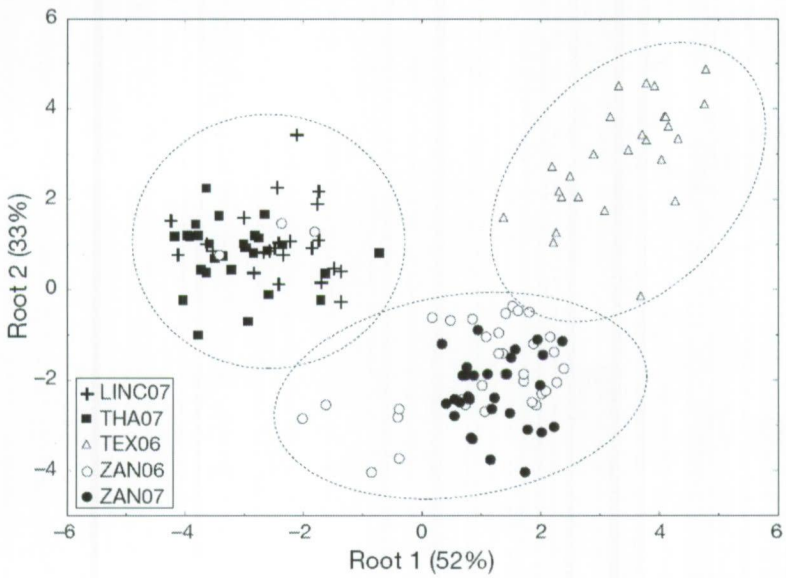


Fig.4 *Solea solea*. Linear Discriminant Function analysis of differences in otolith fingerprints for sole. Three main groups are indicated. Both axes explained 85 % of the variation between groups.

Finally, to test the temporal stability of our fingerprinting approach, the element concentrations for fish sampled in 2006 and 2007 near Zandvliet were compared with a t-test for independent samples. Significant differences were found between the two years for 6 of the 10 elements: Na ($t_{64} = 6.0$; $p < 0.0001$), Mg ($t_{64} = 2.12$; $p = 0.03$), Cu ($t_{64} = 2.08$, $p = 0.04$), Rb ($t_{64} = 6.39$, $p < 0.0001$), Sr ($t_{64} = 8.45$; $p < 0.0001$) and Pb ($t_{64} = 2.59$; $p = 0.01$). Higher levels of these elements were observed in 2006 compared to 2007. Despite these temporal differences, the classification model remained powerful when using temporal samples of ZAN07 as a test group; all individuals were assigned to ZAN06, with high probabilities for all samples (probabilities ≥ 0.97-1.00), suggesting that the temporal variation might be negligible compared to the spatial effect.

Discussion

The discrimination power of otolith microchemistry to define and trace back biological populations is highly dependent on local physico-chemical conditions and on the degree of larval dispersal away from adult spawning habitats. The present study applies otolith microchemistry as a reliable tool for studies of connectivity of *Solea solea* over a wide spatial scale along the coasts of the North Sea and on opposite sides of the southern North Sea. The main result was the high assignment proportion to all four nursery areas, pointing to the spatial differences in juvenile otolith fingerprints.

The elemental concentrations were comparable to published studies in sole and other marine fish, although the values for Zn and Cu were somewhat low (Campana 1999; de Pontual et al. 2000; Vasconcelos 2007). Significant differences in elemental composition between the sampling sites were found for eight of the ten elements analyzed. Strontium concentration was lowest in Zandvliet and highest in the samples from Lincolnshire. Given the strong relationship between Sr and salinity, this was expected as Zandvliet is situated in the brackish part of the Scheldt estuary where salinity is much lower than offshore Lincolnshire (Limburg 1995; Campana 1999; Secor & Rooker 2000). Otoliths from the Scheldt were also characterized by higher Ba concentrations. This elevated Ba signal in estuarine, low salinity nursery areas compared to coastal areas has been observed in other fish species (Hamer et al. 2006; Leakey et al. 2009). Sources of ambient Ba include terrestrial runoff,

groundwater, pollution and remobilisation from sediments (Hamer et al. 2006). Cu and Pb values were also relatively high in the fish from the Scheldt. The Scheldt basin is indeed a highly industrialized area with a history of heavy metal pollution, especially for the elements Cu, Pb, Cd and Zn (Baeyens 1998; Baeyens et al. 2005). The high Mn concentrations measured in the samples from Texel may be the result of the enrichment of the Wadden Sea in both dissolved and particulate Mn compared to the German Bight or the North Atlantic Ocean. Especially in summer, Mn concentrations reach values ten times higher than in winter due to the increased microbial activity and reducing conditions in the tidal flat sediments (Dellwig et al. 2007).

Trace elemental composition of sole otoliths are appropriate natural tags of these nursery areas in the North Sea; based on 6 of the 10 elements measured (Mn, Ba, Sr, Rb, Na and Mg), 88 % of the juvenile sole were correctly assigned to their site of origin. Microchemical variation was higher between samples on opposite sides of the southern North Sea than between samples along the same coastline. Both English sites (Thames and Humber) clustered together and differed more from the samples of Zandvliet and Texel. Although sole from different geographical locations and latitudes differ in life history traits and biological features, our results agree with otolith microchemistry studies on sole carried out in other systems, such as the Bay of Biscay (de Pontual et al. 2000), the Portuguese coast (Vasconcelos 2007) and the Thames (Leakey et al. 2009). De Pontual et al. (2000) showed that spatial discrimination based on otolith microchemistry was possible for juvenile sole in two estuaries in the Bay of Biscay, separated by a distance of about 200 km. A relatively high classification success (70 %) has been observed for juvenile sole caught in eight estuarine nursery areas along the Portuguese coast (Vasconcelos 2007). Finally, Leakey et al. (2009) observed differences in sole elemental signature between estuarine and coastal habitats in the Thames estuary.

In a highly dynamic system such as the North Sea (Otto et al. 1990; Grioche et al. 2001), connectivity between populations in marine species with a pelagic larval phase is expected to be high. Prevailing currents may potentially transport larvae over long distances (Caley et al. 1996). Nevertheless, coastal marine populations may not be as open as thought and the level of connectivity might be overestimated (Cowen et al. 2000; Cowen & Sponaugle 2009).

Several factors increase the likelihood for a certain level of discreteness in *Solea solea*. Populations of sole have been known to show a lower dispersal capacity than other flatfishes (Rijnsdorp et al. 1992; van der Veer et al. 2000; Grioche et al. 2001), increasing the likelihood for successful population assignments using otolith microchemistry. Unlike the Bay of Biscay, where adults spawn offshore, in the North Sea, spawning takes place in shallow coastal areas and the nurseries are found nearby, along the shallow coasts and in the estuaries. Studies on sole larval distribution suggested that larvae reach the nursery grounds through selective tidal transport (Amara et al. 2000; Grioche et al. 2001). Spawning behaviour of adults appears to be linked to hydrodynamics; they spawn there where the probability of larvae reaching the nurseries is highest (Grioche et al. 2001). Previous studies on sole using other markers have also suggested a limited juvenile dispersal. Stable isotopes pointed to high site fidelity in 0-group juveniles, but an increased mobility in 1-group sole in Portugal (Vinagre et al. 2008b). Low mobility of young sole after settlement was also shown in the Thames estuary based on the consistent relationships between stable isotope signals of juvenile sole and their prey (Leakey et al. 2008). Tagging studies confirmed a similar pattern of limited movement of juveniles and site fidelity (Burt & Millner 2008). The low mobility during the juvenile period explains the success of otolith elemental composition from the juvenile area of the otolith as a tag of nursery grounds.

The observed spatial differences between sole nurseries result most likely from a combination of environmental, biological and ontogenetic factors. The North Sea receives the runoff from many large estuaries and rivers. Estuaries are often exposed to a range of anthropogenic pressures (e.g. dredging, sewage input, domestic and industrial effluents) and are also characterized by differences in temperature regime, hydrodynamics, geology, weathering rates and river flow. All these factors lead to different ambient elemental concentrations among locations and ultimately in the observed differences in otolith composition (Thorrold et al. 2007). Given the positioning of our sampling sites, all in the vicinity of an estuary or river, it was expected that the chemical composition of the otoliths would differ among juvenile sole. Ontogenetic and physiological effects are known to influence otolith composition (Thresher 1999; de Pontual et al. 2003). This was observed in *S. solea* where a decrease in Sr/Ca ratio has been linked to high growth rates during metamorphosis (de Pontual et al. 2003). Additionally, a relationship between reproductive

status and otolith composition was observed for other species (Thresher 1999). Factors such as temperature variation, ontogeny and stress might thus play a role in the spatial variation, prompting for strong background knowledge when performing such studies. In our system, water temperatures were similar between all sites and thus unlikely to be the major cause of the observed Sr variation. Although the soles caught near the Humber consisted mostly of 1-group fish compared to the other sites where only 0-group fish were caught, all fish belonged to the same ontogenetic stage (i.e. non-mature juvenile fish). Consequently, this age difference might only represent a small, if any, contribution to the observed variation in microchemistry. Overall, otolith fingerprints show great applicability as natural tags of migration history, although further investigations are needed to unveil the complex mechanism behind observed microchemical differences (Gillanders & Kingsford 2000; Thorrold et al. 2001).

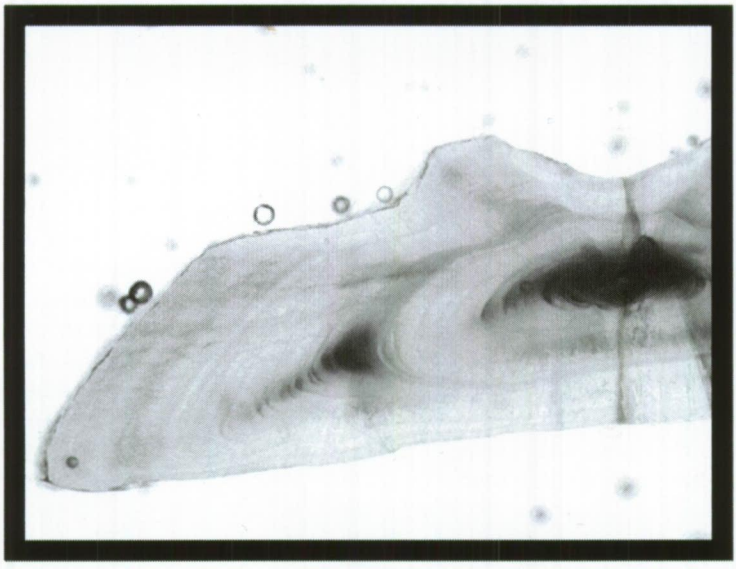
Knowledge on the temporal stability of the geographic variation in fish otoliths is essential for reconstructing individual migration histories. In marine fish, interannual differences in otolith chemistry have been observed, potentially confounding spatial differences. In some cases temporal variability was high, but in other cases spatial trends were similar among cohorts (Gillanders 2002b; Patterson et al. 2008; Schaffler & Winkelman 2008). Here, the elemental fingerprints of sole caught in the Scheldt estuary were significantly different between years. The discriminant analysis, however, indicated that the spatial signal was stable and more important than the temporal differences. Ideally, a standardized library of elemental fingerprints should be built up over time for each estuary (Gillanders 2002b). In reality this information is often lacking and the subsequent assignment of adults to their nursery of origin should be done with care. If temporal variation is obvious, assignment should only be done for year-classes where a juvenile signature has been identified. Although our study did not include all potential nursery areas for sole, our results show great promise for the use of otoliths as natural tags of North Sea nurseries. Future assignment and full connectivity studies, should increase the number of nurseries, include more temporal samples and use appropriate statistical tools (such as individual based Bayesian MCMC algorithms) without *a priori* population information, when not all source populations are sampled (White et al. 2008).

Certain elements, especially heavy metals that are present in trace concentrations, may still represent valuable tracers of distinct environments even though they might be difficult to detect (Arslan & Secor 2005). Therefore it is important to develop a robust method for including these values in statistical analyses and discriminant models. One method proposed is to replace values below the LODs with estimated values, derived from maximum likelihood modeling (Helsel & Hirsch 2002). Another method would be to code such values as present/absent, and to treat these elements as categorical values within multivariate analyses, as established in community ecology studies.

This study is the first step towards assessing patterns of connectivity and the importance of nursery habitats for sole in the North Sea. The next step will be to collect adult otoliths from various regions and attempt to match the chemical signatures of the juvenile portion of their otolith with the atlas generated from juveniles caught on the nursery grounds. Combining information on the relative abundance of sole on each nursery ground with the proportion of adults that show a given nursery signature could quantify the relative importance of particular nurseries for population replenishment. Such information is important for sustainable fisheries management.

Acknowledgements

Research has been funded by the European Community's Seventh Framework Programme under contract n° KBBE-212399 (FishPopTrace). The authors thank L. Bolle (IMARES), S. Geldof (K.U.Leuven), scientists from ILVO and the crew of RV *Belgica* and RV *Zeeleeuw* for their help at sea. Many thanks to O. Tumyr and J. Kosler (Centre of Geobiology, UiB) for their help with the operation of the laser and ICPMS. Acknowledgements to the BeNCoRe network and Research Foundation-Flanders (FWO-Vlaanderen) for a travel grant. E.C. acknowledges a PhD grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). G.E.M. is a post-doctoral researcher funded by the Research Foundation-Flanders (FWO-Vlaanderen). We acknowledge the helpful comments of four anonymous reviewers.



Chapter 3

Combined analysis of otolith microchemistry and shape to detect regional stock structure in sole (*Solea solea*)

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ABSTRACT

Natural tags, such as otolith microchemistry and otolith shape, have become important contributors to understand the migration history and connectivity of populations. Contrasting results between both techniques suggest that the discrimination power of each technique is species-specific and linked to growth rate and environmental variation. Here, we applied otolith microchemistry and shape analysis to sole (*Solea solea* L.) in the Northeast Atlantic Ocean to assess their power in discriminating distinct population units at a regional scale. Trace element composition of otolith core and edge were analyzed in adult sole collected at spawning grounds in the North, Irish and Celtic Seas, the Skagerrak and Kattegat. Significant spatial differences in the chemical composition of the otolith edge were observed, resulting in a global classification success of 55 % to the respective capture location (ranging from 28 % to 88 %). Assignment success of individuals to their capture site was especially high for the Kattegat/Skagerrak region, based on microchemistry fingerprints. Within the North Sea, the assignment success was moderate, suggesting a higher degree of movement of fish between locations or a lack of resolution power. Although differences in otolith shape were found between the various locations, the assignment power to the original location based on shape was lower (40 %) than microchemistry. The combined analysis of otolith microchemistry and otolith shape seems to represent a powerful tool for traceability (total assignment success of 71 %), enabling the future tracking of landed fish from at least three main areas in the NE Atlantic Ocean (Baltic region, North Sea and Celtic/Irish Sea) for fisheries enforcement.

INTRODUCTION

Even though genetic differences between individuals form the basis for reproductive isolation, the probability of detecting significant genetic population structure in marine fish is often small due to the large effective population size and high level of connectivity (Hellberg et al. 2002). Complementary information from alternative methods has turned out to be valuable to identify distinct 'populations' (Palumbi 2004b; Fromentin et al. 2009). Otolith microchemistry (Campana et al. 1994; Jonsdottir et al. 2006b) and otolith shape (Campana & Casselman 1993; Jonsdottir et al. 2006a), parasite load (Charters et al. 2010) and morphometrics (Turan 2004) have been successful in population discrimination.

The discrimination of populations using otolith microchemistry does not necessarily imply genetic differences and therefore the term 'population' does not have a genetic basis. Nevertheless, information from otolith microchemistry can provide important complementary information regarding natal habitats and stock structure (Miller et al. 2005, Feyrer et al. 2007). Due to the unique properties of otoliths (metabolically inert, continuous growth, incorporation of trace elements from the environment), their chemical composition can indeed be used to discriminate among fish that spend part of their life in different environments (Campana 1999; Campana et al. 2000). More specifically, the chemical composition of the otolith core may serve as a marker of the larval and nursery ground environment of an individual because this material is laid down during early life (Campana 1999). Hence, fish spawned at different sites would show a different elemental composition in their core, reflecting distinct spawning units (Thorrold et al. 2001; Warner et al. 2005). The fingerprint of the otolith edge on the other hand, represents a marker of the capture site (de Pontual et al. 2000; Ashford et al. 2005; Vasconcelos 2007). The edge signal, depending on the precision of the technique for measurement, represents the recent days or months before capture (M-Y Chang, pers. Comm., University of Bergen).

Another tool often used as an indicator of population identity is otolith shape. Otolith shape is not only species-specific (L'Abée-Lund 1988, Campana and Casselman 1993), also within a single species, shape may vary between populations (Campana & Casselman 1993). Even though several environmental and biological factors may explain much of the variation in otolith shape, at least part of the variation may hold a genetic basis (Cardinale et al. 2004).

In general, variation in shape indicates a prolonged separation of individuals from distinct environments (Jonsdottir et al. 2006a). Fourier analysis of otolith shape is the most commonly used method for population delineation (Campana & Casselman 1993; Gonzalez-Salas & Lenfant 2007; Mérigot et al. 2007; Stransky et al. 2008).

Sole (*Solea solea* L.) is a commercially important flatfish occupying the coastal waters of the North-East Atlantic Ocean and Mediterranean Sea. The main spawning grounds in the North Sea are situated in the inner German Bight, in the Eastern English Channel, off the Belgian coast, off the Thames and on the Norfolk Banks (De Clerck & Van de Velde 1973; Borremans 1985; Rijnsdorp et al. 1992; Wegner et al. 2003). Depending on the water temperature, sole spawn between January and April in the Bay of Biscay (Koutsikopoulos & Lacroix 1992), from February to April in the Celtic Sea, from April to June in the Irish Sea (Symonds & Rogers 1995) and from early April until August with a main peak in May in the North Sea (Van der Land 1991; Rijnsdorp et al. 1992). Currently, population genetic studies have suggested low genetic differentiation among Atlantic sole populations, following an isolation-by-distance model and larger vicariant differences between the Atlantic Ocean and Mediterranean Sea (Kotoulas et al. 1995; Exadactylos et al. 1998; Exadactylos et al. 2003; Rolland et al. 2007). In the Atlantic, latitudinal differences in life history traits have been observed (Rijnsdorp & Vingerhoed 1994, Witthames et al. 1995). Fecundity increases with latitude for a 35 cm female, from 200 000 eggs near Portugal to 450 000 eggs in the southeastern North Sea. Also fecundity differences exist between the western (Flamborough) and eastern part of the North Sea (German Bight). Furthermore, there are differences in egg size, total length, age and size at maturity among groups; all of which could suggest the existence of discrete populations.

Results of otolith microchemistry studies on *Solea solea* have also indicated differences consistent with separate populations based on the discrimination of juveniles caught in nurseries in the North Sea (Leakey et al. 2009), in the Bay of Biscay (de Pontual et al. 2000) and along the Portuguese coast (Vasconcelos et al. 2008). Most of these studies have been done in the context of estuarine immigration and connectivity among nursery grounds, while to our knowledge only one study has included adult individuals (Vasconcelos et al. 2008). Otolith shape was also found to differ between sole captured at different locations in the

Gulf of Lions (Mediterranean Sea) (Mérigot et al. 2007). Although these studies have all been confined to rather small geographic areas, their results indicate that otolith analyses are a useful tool for the study of sole population structure.

The major aim of this study was to compare the combined discriminative power of otolith microchemistry and otolith shape to identify different population units of adult sole in Northern Europe. Additionally, the chemical composition of the otolith edge was determined to assess the traceability power to fishing grounds and the composition of the otolith core was analyzed to detect distinct spawning units.

MATERIALS AND METHODS

Sampling

Adult sole were collected during research surveys in spring or autumn at seven spawning grounds in the North Sea, Celtic Sea, Irish Sea and the Skagerrak and Kattegat, in 2007 and 2008. The spawning population of the Belgian coast was sampled during both years; all other locations were sampled once (Fig.1, Table 1). Sagittal otoliths were extracted onboard or in the lab and kept in trays or eppendorf vials. From each location, about 30-40 otoliths were selected for otolith microchemistry, resulting in a total of 244 otoliths. Fish of similar length were chosen in order to minimize any size effects. Left sagittae were used for the microchemistry analysis, except when only the right sagitta could be retrieved. Given that asymmetry between right and left otoliths in sole has been shown (Mérigot et al. 2007), only left otoliths (N = 188) were selected for the shape analysis.

Otolith microchemistry

Sample preparation

To remove surface contamination, the otoliths were cleaned using a standard decontamination protocol (Otolith Research Laboratory, Bedford Institute of Oceanography, Canada). Only ultraclean chemicals and equipment made of teflon, polyethylene or polypropylene were used. Otoliths were cleaned from any adhering tissues, placed in labelled vials, covered with ultrapure water and sonified for 5 min. Then otoliths were scrubbed with a nylon brush, triple rinsed with ultrapure water, placed back in the vials and

sonified for another three minutes. After cleaning, the otoliths were dried inside the vial. The dry otoliths were weighed to the nearest 0.005 mg and then stored in acid washed vials awaiting further preparation. Next, otoliths were embedded in epoxy resin (NM Laminering 275A, Nils Malmgren AB). A transverse section containing the otolith core was cut using a low speed diamond saw. Sections were attached with resin onto glass slides and the otoliths were ground with a series of grinding papers and polished with diamond paste (3 µm - 1 µm - 0.25 µm) until the core was reached. Specimens from different locations were randomized on each slide to avoid preparation artefacts. Finally, to remove any remaining surface contamination, each slide was sonified for 10 min in ultrapure water and dried under a laminar flow.

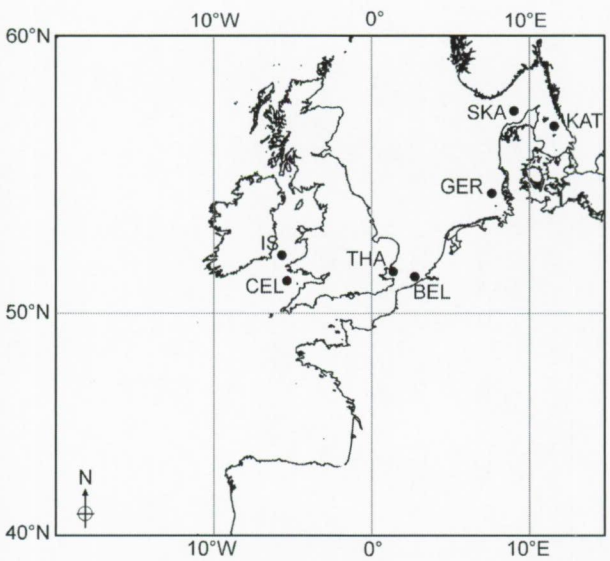


Fig.1 Map of sampling area showing collection sites of *Solea solea*.

Table 1 Sample information for *Solea solea*: Sample code, code corresponding with Fig.1 (Map), sampling location, sampling year (Asterisks (*) indicate if the sample is taken during the spawning season) and month, position, number of otoliths analyzed for microchemistry (N_{mc}) and shape (N_{shape}), Total fish length TL (mean ± standard deviation); otolith weight OW (mean ± standard deviation). Numbers of males (M) and females (F), NA = not available (sex unknown).

Sample code	Map	Location	Year	Month	Latitude	Longitude	N_{mc}	N_{shape}	TL (cm)	OW (mg)	M	F
KAT07	KAT	Kattegat	2007	Nov	57°08'91"N	11°38'52"E	35	35	27.00 ± 1.87	16.35 ± 4.07	17	18
SKA07	SKA	Skagerrak	2007	Nov	58°09'43"N	9°30'32"E	22	22	28.50 ± 3.86	16.83 ± 6.68	7	15
GER07	GER	German Bight	2007*	May	54°31'12"N	7°53'23"E	34	21	25.43 ± 4.45	10.43 ± 4.53	12	22
BEL07	BEL	Belgian Coast	2007*	May	51°21'16"N	2°56'11"E	26	24	26.29 ± 3.86	11.57 ± 4.43	NA	NA
BEL08	BEL	Belgian Coast	2008*	May	51°23'22"N	3°10'01"E	30	27	26.98 ± 0.73	13.15 ± 2.63	8	22
THA07	THA	Thames	2007	Aug	51°27'80"N	1°20'00"E	35	28	26.77 ± 1.57	13.37 ± 3.32	5	10
CEL08	CEL	Celtic Sea	2008*	Apr	50°49'00"N	5°01'00"W	32	19	26.20 ± 0.91	16.26 ± 2.78	31	1
IS08	IS	Irish Sea	2008*	Mar	52°13'00"N	5°20'00"W	30	12	26.92 ± 1.24	16.43 ± 3.32	21	9
Total							244	188			101	97

ICPMS measurements

LA-ICPMS analyses were carried out using a Finnigan ELEMENT 2 ICPMS (Thermo Electron Corporation, Bremen) coupled to a UP266 MACRO laser system (New Wave Research). Laser conditions (10 Hz repetition rate, 50 ms dwell time, medium resolution mode) induced a crater of 60 µm diameter at two zones on the otolith: one in the core region (C) and one at the edge (E). The core spot corresponds to the larval period before metamorphosis, integrating approximately the first 15 days after hatching (see Lagardère & Troadec 1997). The spots were always taken along the dorsal axis. The following isotopes were measured: ^{7}Li, ^{23}Na, ^{24}Mg, ^{43}Ca, ^{55}Mn, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{85}Rb, ^{86}Sr, ^{111}Cd, ^{138}Ba and ^{208}Pb. Counts per second were processed using the software GLITTER (GEMOC, Macquarie University) with ^{43}Ca selected as internal standard. Standard reference materials (NIST612, NIES-22 (National Institute for Environmental Studies, Japan)) were measured after every 15 spots. The calculations of the element concentrations were made based on NIST612 for the elements Li, Mn, Co and Rb; and NIES-22 for all other elements. The minimum detection limit at the 99 % confidence level was calculated by GLITTER using an algorithm developed by Longerich et al. (1996). Data below the limits of detection were set to zero (Helsel & Hirsch 2002).

Data analysis

Data were visually inspected for outliers using Cook's distance and outliers were removed from the dataset. Fish length ($\log_{10}$ transformed) was compared among sampling locations with a univariate ANOVA. Concentrations of Na, Mg, Sr, Mn and Ba were $\log_{10}$ transformed; Cu, Zn, and Rb were $\log_{10}(x+1)$ transformed and for Cd and Co a square root transformation was applied, in order to achieve normal distributions and homogenous variances between groups. Analyses of population patterns were performed separately for the otolith core and edge data, and comparison of core and edge data within individuals was made using a t-test for dependent samples because these measurements were made on the same fish.

- Edge data

First, differences in otolith chemistry among sampling locations were analysed with an analysis of covariance (ANCOVA), with fish length as covariate. Because there was no significant length effect, comparisons of element fingerprints among locations were done using multivariate and univariate analysis of variance (MANOVA). *Post-Hoc* Tukey HSD tests were applied to determine pairwise comparisons among fishing locations. Secondly, a

forward stepwise linear discriminant function analysis (LDFA) was performed on the edge data, to evaluate the use of multi-element fingerprints of otolith edge for classifying fish to fishing grounds (all locations separately) and to the three main regions: 'Baltic region' (i.e. KAT, SKA), 'North Sea' (i.e. BEL, THA, GER) and 'Irish/Celtic Sea' (i.e. IS, CEL). A cross-validation algorithm using a jackknife technique was used to determine the classification accuracy. We applied a randomisation technique to test the significance of the observed reclassification success compared with that expected by chance (White & Ruttenberg 2007).

- Core data

Fish sampled at a single spawning ground do not necessarily originate from the same spawning ground, which makes LDFA and ANOVA procedures less appropriate (Arkhipkin et al. 2009). To determine whether fish caught at different locations also showed distinct chemical signatures of their core, data were analyzed with a Principal Component Analysis based on the correlation matrix and the results are presented in a biplot. Independent agglomerative hierarchical cluster analysis was done on the transformed data to verify results from the PCA, using Ward's procedure, based on Euclidean distances between individuals.

- Temporal variation

To assess temporal variation, the samples BEL07 and BEL08 were compared with a t-test for independent samples. Only edge composition was compared because fish sampled on the Belgian Coastal banks over the two years did not necessarily share the same natal origin and could thus differ in core composition.

Otolith shape

Otolith images were acquired using a camera mounted on a dissecting microscope. The images were taken with optimal magnifications depending on the otolith size and analyzed with the ImageJ v.1.43 software (Rasband 1997-2009). Spatial calibration to real linear (mm) units was achieved using a reference micrometer image taken at the same magnification during each session.The otolith image was converted to an 8-bit image and the contrast was optimized. Next, the otolith outline was detected and the following set of shape descriptors was calculated: width (W), height (H), otolith area (A), Perimeter (P) and Feret diameter (FD). FD is the longest distance between any two points along the selection boundary. Using these variables, four derived shape indices were calculated. These included circularity (Circ),

roundness (Round), ellipticity (Ellipt), rectangularity (Rect) and form. Circularity was calculated in ImageJ using the formula: *circularity* = $(4\pi^*A)/P^2$. A value of 1.0 indicates a perfect circle; as the value approaches 0.0, it indicates an increasingly elongated shape. Roundness also provides information on the similarity to a perfect circle, but is based on the ratio between the area and the major axis. Ellipticity indicates whether changes in the axes are proportional and rectangularity describes length and width variation, with a value of 1.0 similar to a perfect square (Tuset et al. 2003). Form measures surface area irregularity; values < 1.0 are irregular. These indices are calculated from Tuset et al. (2003).

The shape of each otolith was also described based on Elliptic Fourier Descriptors (EFD). Forty harmonics were calculated for each otolith based on the longest radius. Each harmonic is characterized by four coefficients, resulting in 160 variables. The higher the number of harmonics, the greater the accuracy of the outline description (Kuhl & Giardina 1982). The oftware SHAPE v.1.3 was used to calculate the EFDs, invariant to otolith size and orientation (Iwata & Ukai 2002).

Data analysis

- Shape indices

All variables were $\log_{10}$ transformed to meet the assumptions of normality and homogeneity of variance. Any effects of fish size were corrected using otolith weight as the standardizing variable following the equation $M_{STD} = M_o\ (OW/OW_o)^b$ (Lleonart et al. 2000). With M_o the original measurement, M_{STD} the weight-adjusted measurement, OW the mean otolith weight from all samples (14.32 mg in our study), OW_o the otolith weight, b slope of the regression of $\log_{10}M$ on $\log_{10}OW$ using all specimens. To check for redundancy among the shape variables, Pearson correlations were calculated among variables. After final selection of the variables, the standardized measurements were compared between the locations using a one-way ANOVA.

- Fourier parameters

A PCA was carried out on the normalized EFD's to summarize the information of the variation contained in the high number of coefficients. The coefficients corresponding to the first harmonic were excluded because they were constant and not relevant to the shape analysis. The PCA was done with the program PRINCOMP in the SHAPE software (Iwata & Ukai

2002). Next, the normalized EFD's were combined with the selected shape indices circularity, rectangularity, ellipticity and standardized Feret's diameter in a forward stepwise linear discriminant function analysis to describe the discrimination success of samples based on otolith shape. A cross-validation algorithm was used to determine the classification accuracy and a randomisation technique was applied to test the significance of the observed reclassification success compared with that expected by chance (White & Ruttenberg 2007).

- Combined analysis otolith shape and microchemistry

Finally, a forward stepwise linear discriminant function analysis was done including both shape variables and element concentrations measured at the otolith edge. A cross-validation algorithm was used to determine the classification accuracy.

Statistical analyses were done with the software STATISTICA 9.0 (StatSoft, 2009) and SAS v.9.1 (SAS Institute); significance level used for all tests was $\alpha < 0.05$.

RESULTS

Otolith microchemistry

Concentrations of Li and Pb were below the limit of detection (LOD) in more than 30 % of the samples; therefore these elements were removed from the dataset (Supplementary materials, Table S1). Ten elements (Na, Mg, Mn, Co, Cu, Zn, Rb, Sr, Cd, Ba) were measured at detectable levels and were included in the statistical analyses. For most elements, the mean percentage of data below the LOD was lower than 10 % except for the elements Co, Cu, Zn and Rb (Supplementary materials, Table S1). The most abundant trace elements (concentration < 100 $\mu g.g^{-1}$) measured in the sole otoliths were Mg (mean concentration of 19.24 $\mu g.g^{-1}$), Mn (mean concentration of 10.91 $\mu g.g^{-1}$) and Ba (mean concentration of 2.68 $\mu g.g^{-1}$) (Supplementary Materials, Table S2). Element concentrations differed significantly between core and edge for Na ($t = 22.88$; $p < 0.001$), Mg ($t = 31.89$, $p < 0.001$), Co ($t = 28.58$, $p < 0.001$), Mn ($t = 15.75$, $p < 0.001$), Cu ($t = 6.18$, $p < 0.001$), Zn ($t = 2.75$, $p = 0.006$), Cd ($t = 14.15$, $p < 0.001$) and Ba ($t = 11.13$, $p < 0.001$). The elements Sr and Rb did not differ significantly between the two otolith zones. Concentrations of most elements decreased significantly from core to edge, except for Cd, which showed the highest concentration in the edge (Fig.2, Table S2).

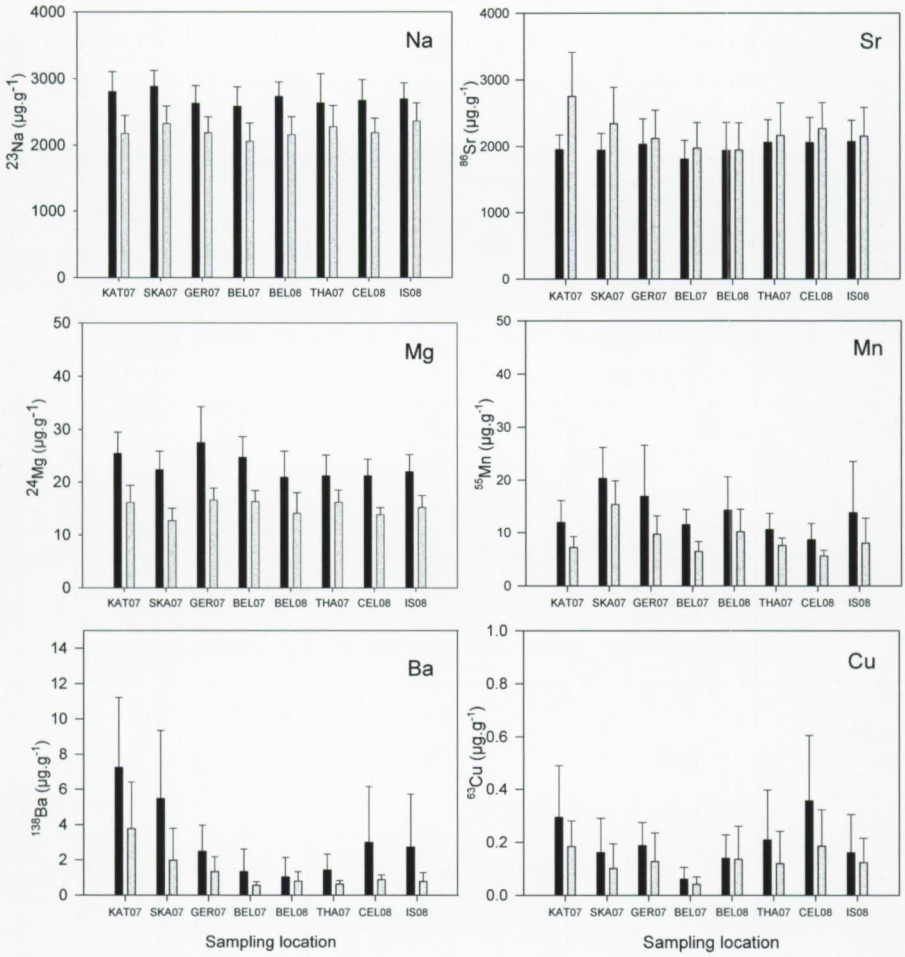


Fig.2 *Solea solea*. Element concentrations (µg.g^{-1}) in otolith core (black bars) and edge (grey bars) according to their sampling location. For sample codes, see Table 1.

- Otolith edge

The multi-element fingerprint of otolith edge was significantly different among samples (MANOVA, $F_{70,1295} = 10.0$, $p < 0.0001$). Significant spatial differences in mean concentration were found for all elements but Zn (ANOVA df = 7, 230: Na: F = 3.74, p = 0.0007; Mg: F = 9.51, p < 0.0001; Sr: F = 8.93, p < 0.0001; Cu: F = 4.29, p < 0.0001; Cd: F = 6.84, p < 0.0001; Ba: F = 53.53, p < 0.0001; Mn: F = 20.35, p < 0.0001; Co: F = 4.36, p = 0.0001; Rb: F = 4.02, p = 0.0003). *Post hoc* tests revealed that up to five elements were significantly different

among locations with Mg, Mn and Ba differing frequently among sampling locations (Table 2). The Irish Sea was not significantly different from the Celtic Sea or from the Thames. The samples caught in the Skagerrak and Kattegat were quite distinct from the rest (Table 2).

Table 2 Results of the Tukey HSD tests for comparisons of otolith edge microchemistry among samples of sole. Filled boxes indicate significant differences. For sample codes, see Table 1.

	Na	Mg	Mn	Co	Cu	Zn	Rb	Sr	Cd	Ba	TOTAL
SKA07-KAT07											5
SKA07-GER07											4
SKA07-BEL07											5
SKA07-BEL08											2
SKA07-THA07											4
SKA07-CEL08											3
SKA07-IS08											5
KAT07-GER07											3
KAT07-BEL07											3
KAT07-BEL08											4
KAT07-THA07											2
KAT07-CEL08											3
KAT07-IS08											2
GER07-BEL07											2
GER07-BEL08											5
GER07-THA07											1
GER07-CEL08											3
GER07-IS08											1
BEL07-BEL08											3
BEL07-THA07											1
BEL07-CEL08											4
BEL07-IS08											2
BEL08-THA07											1
BEL08-CEL08											1
BEL08-IS08											1
THA07-CEL08											4
THA07-IS08											0
CEL08-IS08											0
TOTAL	3	12	15	4	3	0	5	6	9	17	

These patterns were confirmed by the LDFA analysis, which resulted in an overall jackknife reclassification success of 55 %. This was significantly better than the 12 % reclassified by chance ($p = 0.0001$) (Table 3). All elements were entered in the model, with Ba and Mn being the most important contributors to the separation, followed by Mg, Cd, Na, Co, Rb, Cu, Zn and Sr in order of importance. The highest level of correct classification was found for the

samples from the Kattegat (88.24 %) and the Celtic Sea (71.0 %) (Table 3). Misclassifications from the Celtic Sea were mostly done to the Irish Sea sample (16 %). The Irish Sea samples had a very low classification success (28.6 %) although 21 % of the misclassifications were done to the neighboring sample of the Celtic Sea. Samples from the North Sea showed a large overlap and moderate or low assignment score to the original location but most misclassifications were done to other North Sea sampling locations (Fig.3A, Table 3).

Table 3 Jackknife reclassification successes of the linear discriminant function analysis based on otolith edge microchemistry (upper matrix), otolith shape variables (middle matrix) and combined analysis (lower matrix) of sole. Classifications to the correct sampling location are in bold. Grey boxes indicate three regions: Baltic region, North Sea and Celtic/Irish Sea. Classification successes to regions are indicated in last column. For sample codes, see Table 1.

		Assigned location								
		KAT07	SKA07	GER07	BEL07	BEL08	THA07	CEL08	IS08	region
	otolith microchemistry									
Sampling location	KAT07	**88.2**	0.0	2.9	0.0	0.0	0.0	8.8	0.0	88.2
	SKA07	9.1	**68.2**	0.0	0.0	18.2	0.0	4.6	0.0	77.3
	GER07	6.1	3.0	**36.4**	18.2	9.1	15.2	6.1	6.1	78.8
	BEL07	0.0	0.0	7.7	**69.2**	0.0	19.2	3.9	0.0	96.2
	BEL08	3.3	16.7	0.0	20.0	**33.3**	0.0	10.0	16.7	53.3
	THA07	0.0	0.0	11.8	17.7	11.8	**44.1**	2.9	11.8	85.3
	CEL08	3.2	0.0	0.0	3.2	6.5	0.0	**71.0**	16.1	87.1
	IS08	3.6	14.3	7.1	10.7	7.1	7.1	21.4	**28.6**	50.0
	Total									55.0
	otolith shape									
Sampling location	KAT07	**54.6**	6.1	6.1	3.0	9.1	9.1	6.1	6.1	60.6
	SKA07	5.6	**38.9**	11.1	16.7	11.1	0.0	5.6	11.1	44.5
	GER07	0.0	0.0	**40.0**	13.3	0.0	26.7	20.0	0.0	80.0
	BEL07	13.6	4.6	13.6	**40.9**	18.2	0.0	4.6	4.6	72.7
	BEL08	11.1	7.4	3.7	18.5	**37.0**	7.4	14.8	0.0	66.7
	THA07	14.8	0.0	3.7	3.7	11.1	**40.7**	18.5	7.4	59.3
	CEL08	21.1	5.3	21.1	15.8	10.5	10.5	**15.8**	0.0	15.8
	IS08	0.0	0.0	10.0	0.0	10.0	10.0	20.0	**50.0**	70.0
	Total									40.0
	microchemistry + shape									
Sampling location	KAT07	**87.9**	0.0	6.1	0.0	0.0	0.0	6.1	0.0	87.9
	SKA07	5.9	**52.9**	0.0	5.9	17.7	0.0	11.8	5.9	58.8
	GER07	9.1	0.0	**63.6**	18.2	0.0	9.1	0.0	0.0	90.9
	BEL07	0.0	0.0	9.1	**72.7**	0.0	13.6	0.0	4.6	95.5
	BEL08	3.7	11.1	3.7	7.4	**63.0**	7.4	3.7	0.0	81.5
	THA07	0.0	0.0	7.4	14.8	11.1	**59.3**	3.7	3.7	92.6
	CEL08	0.0	0.0	0.0	0.0	0.0	11.1	**83.3**	5.6	88.9
	IS08	0.0	0.0	0.0	11.1	0.0	0.0	11.1	**77.8**	88.9
	Total									71.0

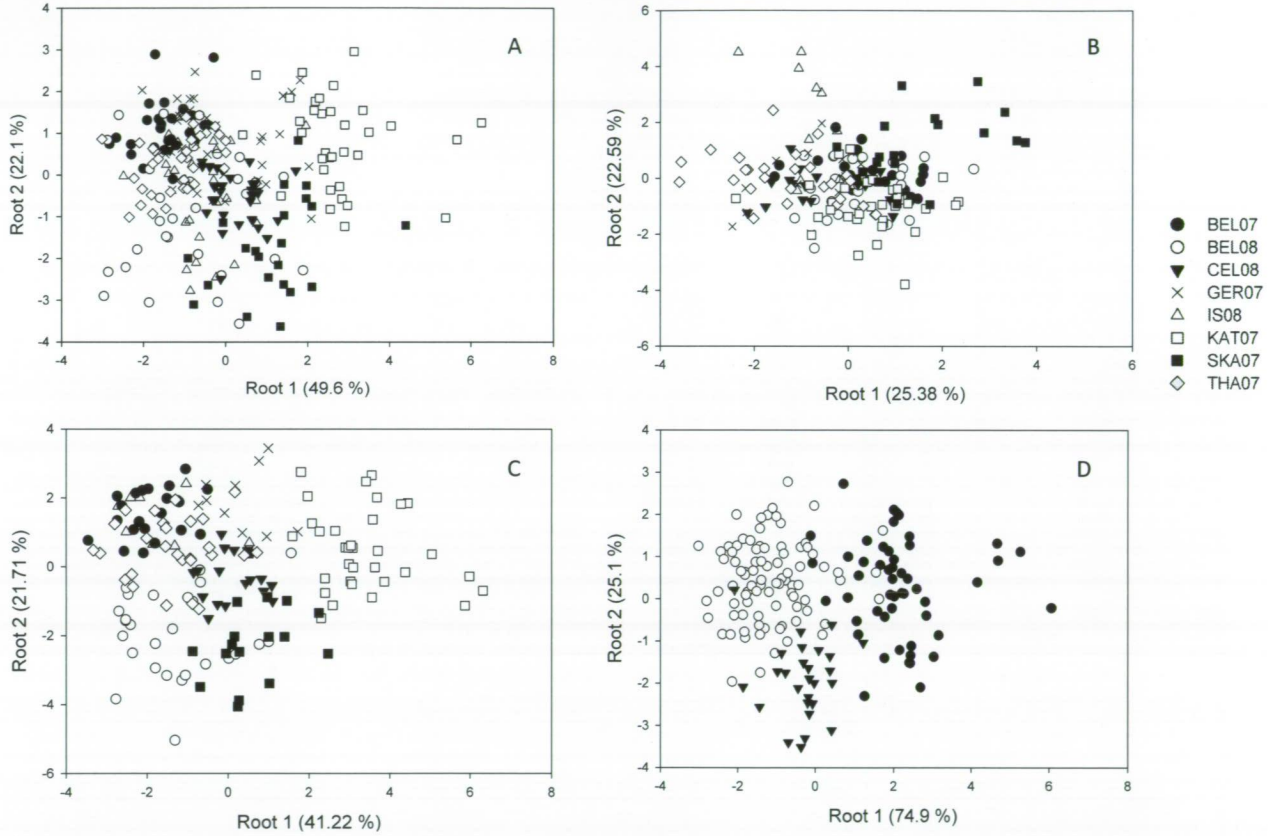
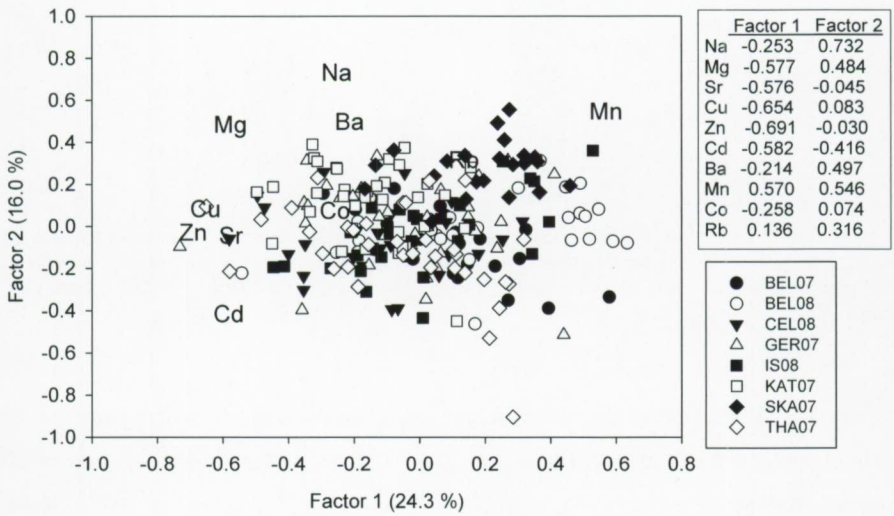


Fig.3 Discriminant Function Analysis of otolith edge microchemistry (A), otolith shape (B), combined microchemistry and shape according to sampling location (C) and combined microchemistry and shape according to larger regions (D). Legend for "A, B and C" is presented to the right. Legend for D: black dots = Baltic region, white dots = North Sea, triangles = Celtic/Irish region. For sample codes see Table 1

The discriminant analysis assigning individuals to one of the three larger regions resulted in an overall assignment success of 78 %: 84 % success was obtained for the Baltic region, 75 % success for the North Sea and 74 % for the Irish/Celtic Sea.

- Otolith core

In general, the PCA showed a great overlap in core data from fish caught at the different locations and there is a large variation within samples. Still, some clustering was obvious for the samples caught in the Skagerrak (SKA07) and the sample BEL08. The first two factors explained only 40 % of the variation observed. Many elements showed similar contributions to the first dimension, while the second dimension is mainly explained by Na, followed by Mn (Fig.4).



	Factor 1	Factor 2
Na	-0.253	0.732
Mg	-0.577	0.484
Sr	-0.576	-0.045
Cu	-0.654	0.083
Zn	-0.691	-0.030
Cd	-0.582	-0.416
Ba	-0.214	0.497
Mn	0.570	0.546
Co	-0.258	0.074
Rb	0.136	0.316

Fig. 4 Principal component analysis based on core microchemistry data of sole. Legend indicates sampling location. For sample codes see Table 1. Factor loadings are presented in the table to the right.

The tree based on Euclidean distances between individuals suggested two large clusters, with the second cluster divided into two smaller clusters. Cluster assignment showed that 94 % of the samples caught in the Kattegat (KAT07) and 91 % of the Skagerrak samples (SKA07) were grouped into the first cluster. The samples caught in the North Sea were predominantly grouped into the second cluster. The samples caught in the Irish and Celtic Sea were found in almost equal proportions in Cluster 1 and 2 (Fig. 5a). If we pooled the

samples in larger geographic regions, the Baltic region grouped for 93 % into Cluster 1 and the North Sea for more than 70 % in Cluster 2 (Fig. 5b).

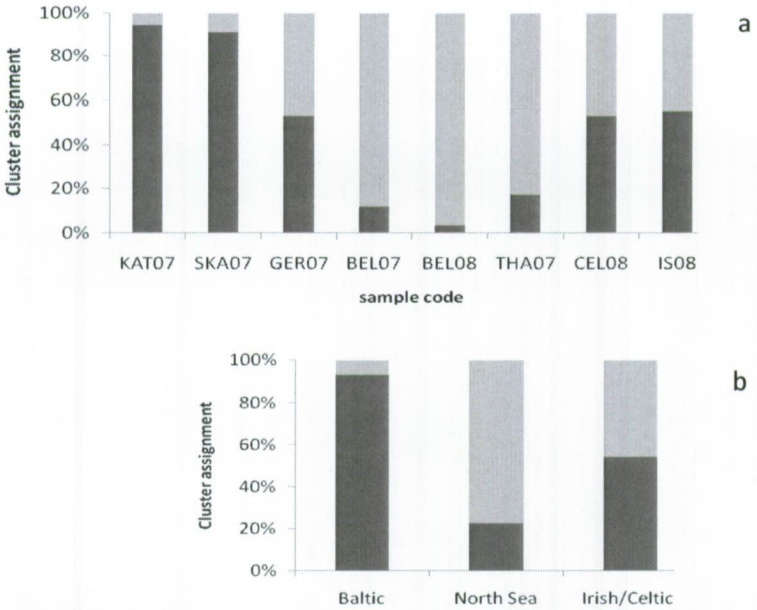


Fig.5 Assignment of sole to clusters (%) based on the core microchemistry composition (Cluster analysis, Ward's method). Individuals with more similar elemental composition are allocated to the same cluster. Assignments according to sampling locations (a) and according to larger regions (b). Dark bars = Cluster 1, Light bars = Cluster 2.

- Temporal variation

There was significant temporal variation in the edge composition of the samples from the Belgian Coast (BEL07 and BEL08). The concentrations of Cu ($t = 2.32$; $p = 0.02$), Zn ($t = 3.69$, $p < 0.0001$) and Mn ($t = 3.84$, $p = 0.0003$) were higher in 2008 compared to 2007, while Cd ($t = 2.08$, $p = 0.04$), Co ($t = 5.56$, $p < 0.0001$) and Mg ($t = 2.86$, $p = 0.006$) had a higher concentration in 2007. The mean concentrations of Sr, Rb, Na and Ba were not significantly different between years. Due to the significant variation, the two samples could not be pooled and were treated as separate samples in all analyses.

Otolith shape

There was a significant positive correlation between fish length and otolith weight ($r = 0.71$, $p < 0.0001$). Otolith weight was also significantly correlated with otolith area ($r = 0.93$, $p < 0.05$), otolith perimeter ($r = 0.92$, $p < 0.05$), Feret's diameter ($r = 0.93$, $p < 0.05$), width

($r = 0.91$, $p < 0.05$) and height ($r = 0.90$, $p < 0.05$). These shape measurements were thus standardized to remove the weight (size) effect. Several shape indices showed redundancy: Area and Perimeter were strongly correlated with Feret's diameter ($r = 0.96$, $p < 0.05$) and therefore eliminated. Otolith width and height were also excluded because they were correlated with multiple other variables. Also the derived indices 'form' and 'roundness' were strongly correlated with several other indices and were thus removed from further analyses. 'Circularity', 'rectangularity' and 'ellipticity' were retained because they were not strongly correlated with other indices.

There were significant differences in shape among sampling locations (MANOVA, $F_{28,632} = 3.15$, $p < 0.0001$). The mean values of circularity ($F_{7,178} = 3.15$, $p = 0.0036$) and FD ($F_{7,180} = 5.6$, $p < 0.0001$) were significantly different among locations, while rectangularity ($F_{7,178} = 0.39$, $p = 0.91$) and ellipticity ($F_{7,178} = 1.02$, $p = 0.42$) were similar among locations. *Post hoc* tests indicated that circularity was different between Irish Sea and Skagerrak/Kattegat (Fig.6) while standardized FD showed significant differences among multiple locations (Fig.6).

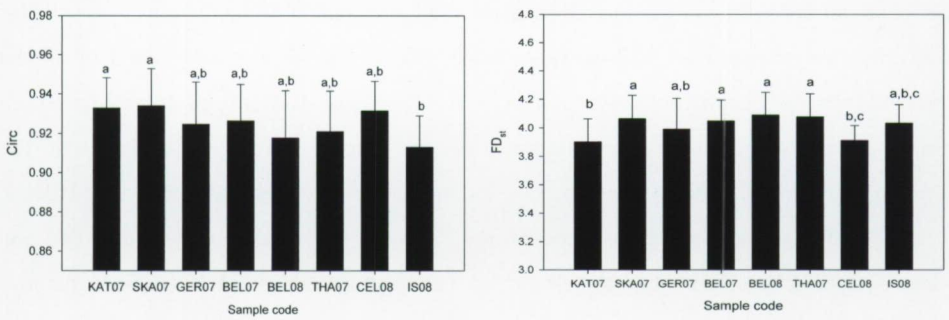


Fig.6 Circularity and standardized Feret Diameter (mean and standard deviation) for all sole samples. Bars labeled with different lowercase letter are statistically different from each other (post-hoc multiple comparison with Tukey HSD test). For sample codes, see table 1.

The first 11 Principal Components explained 94.8 % of the variation in otolith shape and were necessary to reconstruct otolith outlines. The first two principal components accounted for 63 % of the variation in shape. The forward stepwise LDFA model selected 23 out of the 160 variables for incorporation in the model (FD and 22 fourier harmonics). The overall classification success of individuals to their original sampling location, based on these shape variables was however rather low (40 %), but significantly higher than that expected

by chance ($p < 0.0001$). Correct classifications ranged from 15 % (CEL08) to 55 % (KAT07) and were in general lower than classifications based on otolith microchemistry (Table 3).
Twenty percent of the misclassifications from IS08 were done to CEL08. The graphical presentation of the LDFA analysis also confirmed the great overlap in samples from the various locations (Fig.3B).

Combining both otolith microchemistry data and shape variables into one stepwise discriminant model resulted in the selection of 30 variables including 7 element concentrations (Ba, Mn, Cd, Mg, Co, Na, Cu), Feret diameter and 22 Fourier harmonics. The total assignment success (71 %) was much higher than the assignment success based only on microchemistry (55 %) or otolith shape (40 %) (Table 3, Fig.3C). Fish from the three larger regions could clearly be separated based on the combination of otolith shape and microchemistry (Fig. 3D).

Discussion

A good understanding of the migration history and connectivity of populations is crucial for an effective management and conservation of marine fish. This requires first of all the identification of distinct ecological units. Our results demonstrate the usefulness of otolith microchemistry and shape as a traceability tool for sole over a wide spatial scale in the North-East Atlantic Ocean. In general, our findings support the results from genetic studies, namely large spatial differences between the Kattegat, Skagerrak and the North Sea and more subtle differences within the North Sea. Furthermore, otolith shape alone turned out to be less powerful than the chemical composition of otolith edge in assigning fish to their sampling location.

The element concentrations measured in our study were comparable to other studies on *Solea solea* (de Pontual et al. 2000; Vasconcelos 2007; Chapter 2) and also in the same range as other North Atlantic fish such as whiting (*Merlangius merlangus*) (Tobin et al. 2010) and haddock (*Melanogrammus aeglefinus*) (Wright et al. 2010). We observed significant differences in the concentration of most elements between the otolith core and edge. All elements, except for Cd, Sr and Rb, were enriched in the core. The mean concentration of

Mn, for example, was higher in the core region than in the edge in all samples. A Mn peak has been observed in many species and sometimes even used as a measure to detect the core region (Fontes et al. 2009). Enrichment of trace elements in the core region appears a general characteristic in a wide range of fish (Brophy et al. 2004; Ruttenberg et al. 2005; Warner et al. 2005) and it has also been observed in squid statoliths (Warner et al. 2009). The driving mechanism for core enrichment remains unknown, although it has been linked to the large amounts of proteins necessary for initial calcification and the relatively low Ca concentration in the core region, resulting in a high relative concentration of trace elements (Ruttenberg et al. 2005). In order to use otolith chemistry as a population discrimination tool and address connectivity questions, the variations in composition must reflect consistent differences in locations, diet, or physiology. Although it is certain that ontogenetic shifts and maternal investment may lead to an increase of elements in the core, the relative importance of maternal effects and environment remains unclear (Ruttenberg et al. 2005). Still, the use of natal element signatures in the otolith core to examine larval dispersal patterns and connectivity has been demonstrated in several studies (Thorrold et al. 2001; Rooker et al. 2008; Vasconcelos et al. 2008; Fontes et al. 2009). The otolith core is composed of material laid down during early life and thus provides information on environmental differences experienced after hatching and before metamorphosis and settlement in the nursery areas. A study by Lagardère and Troadec (1997) has shown that in sole larvae, the onset of metamorphosis occurs at a sagittal radius of approximately 60 µm when larvae are about 22 days post-hatching. The examination of natal element signatures has already demonstrated the mixed nursery origin of adult sole along the Portuguese coast in a study by Vasconcelos et al. (2008). In our study, two main clusters were identified based on the core composition of adult sole. These clusters may represent distinct spawning units in sole. One cluster contained mainly sole sampled in the Kattegat and Skagerrak, while the other cluster contained samples from the North Sea and Irish/Celtic Sea. At a smaller scale within the North Sea, core chemistry appeared rather similar among sampling locations. The lack of discrimination among the cores from different areas could either indicate similar early life history or a lack in resolution power due to similar environments. The success of using otolith core chemistry as a population discrimination tool appears variable between species (Swan et al. 2006).

The elemental fingerprint of the otolith edge represents a record of the harvest location. Spatial variation in element concentration at the edge was found for almost all elements measured. The high assignment success obtained based on edge microchemistry for individuals collected in the Kattegat, Skagerrak and Celtic Sea, suggests that sole from these areas are rather site-specific. Since the Celtic Sea sole were collected during the spawning season, the elemental fingerprint indicates additionally a distinct spawning population in the Celtic Sea. This result is consistent with results from tagging studies showing that there is only limited movement of sole between the Celtic Sea and adjacent areas; once recruited to an area, sole tend to remain there (Horwood et al. 1993). On the other hand, many of the Irish Sea sole were also assigned to the Celtic Sea, suggesting a great similarity in elemental composition between the neighbouring spawning populations from the Irish and Celtic Sea. This might be explained by the proximity of both sites; the sample from the Irish Sea was taken in the Southeastern part of the Irish Sea, near Cardigan Bay, and the Celtic Sea sole were collected at the main spawning ground, about 150 km south (off Trevose head, UK).

The most important contributors for the discrimination among sampling locations (otolith edge) were the elements barium and manganese. Barium concentration was highest in the Kattegat and Skagerrak samples and much lower in the samples from the North Sea. Trace elements such as Ba and Mn are potentially enriched in inshore coastal waters and sheltered bays; sources of Ba include river runoff, pollution and remobilization from the sediment (Hamer et al. 2006). The enclosed nature of the Skagerrak/Kattegat area is characterized by large river runoff, combined with the influx of lower salinity water from the Baltic Sea (Andersson 1996; Rodhe 1996), which might explain the relatively higher concentration in Ba and Mn. This is consistent with other studies on fish (Longmore et al. 2010) and shellfish (Förlin et al. 1996; Baden & Neil 2003) observing high manganese concentrations in the Skagerrak. The high manganese concentration in tissues is sometimes used as a biomarker for hypoxia (Förlin et al. 1996, Baden & Neil 2003) because hypoxia makes sediment-bound manganese more bioavailable (Middelburg & Levin 2009). In the Skagerrak hypoxia has been reported at some locations (Förlin et al. 1996).

The lack of discrimination between sampling sites within the North Sea based on the otolith edge microchemistry could result either from similar environments or from the migration of individuals between areas. Small scale spatial differences in otolith microchemistry have been found in juvenile and adult sole from the Portuguese Coast (Vasconcelos 2007;

Vasconcelos et al. 2008), the Bay of Biscay (de Pontual et al. 2000) and the Thames (Leakey et al. 2009), suggesting that differences in water chemistry are possible at relatively small spatial scales. Moreover, the high discrimination power observed in juvenile sole from different nursery grounds in the North Sea (chapter 2) and the high number of elements in the current study makes it unlikely that the lack of discrimination within the North Sea is simply due to homogenous environments. On the other hand, if mixing of individuals is high enough, it could mask discrimination among sites. This would confirm the results of population genetic studies, indicating homogeneity and considerable gene flow within the North Sea (Exadactylos et al. 1998; Exadactylos et al. 2003).

Besides spatial variation, also interannual variation in otolith elemental composition was observed for the two samples from the Belgian coast, taken just 20 to 30 km from each other. Temporal variability in otolith microchemistry has been found in a number of studies (Gillanders 2002b; Swearer et al. 2003; Schaffler & Winkelman 2008) and has been attributed to environmental variation, dietary or physiological differences and analytical bias (Schaffler & Winkelman 2008). Fish from both years were of similar size and ontogenetic stage and therefore physiological effects are probably small in our study. Nevertheless, dietary differences have been reported for *Solea solea* as a function of sex, age and season (Molinero & Flos 1991, Molinero & Flos 1992). Even between individuals of the same age, differences in the basic diet existed (Molinero & Flos 1992). Analytical bias was minimized by randomly ordering and analyzing the samples from the different years together, over several runs. Therefore the observed variation most likely resulted from differences in runoff because the Belgian coastal zone is strongly influenced by rivers such as Scheldt, Rhine and Meuse. Temporal replicates were only available at one location, which makes it difficult to test the effects of temporal variation at a larger scale. Interannual variation was observed in otolith concentrations in samples of juvenile sole taken from the Scheldt estuary, although both temporal samples still clustered closely together (chapter 2). The adult sole however, showed larger temporal differences. Due to their higher mobility, adult sole can presumably cover a larger area, experiencing more different environments compared to juveniles. More temporal replicates should be included in future studies to better understand how chemical signatures vary in time.

In addition to differences in microchemistry, the environment may also induce variation in otolith shape. Variation in otolith shape appears strongly linked to differences in growth rate (Campana & Casselman 1993) but can also reflect behavioral differences or differences in diet (Aguirre & Lombarte 1999). In a common garden experiment with Atlantic cod (*Gadus morhua*), Cardinale et al. (2004) showed that genetic differences influenced the shape of the otolith itself, while environmental differences altered the rates of otolith growth, which in its turn modified otolith shape. The fact that, besides environmental effects, different genotypes may induce differences in otolith shape, supports the use of otolith shape as a population discrimination tool (Cardinale et al. 2004). Some studies showed higher power using shape morphology (Campana & Casselman 1993; Campana et al. 1994), others have found a higher assignment success using otolith microchemistry (Longmore et al. 2010) or considered both techniques similar (Turan 2006).

Even though in our study, significant differences in shape variables among sampling sites were found, the assignment power based on the shape variables was lower than 55 % for all sampling sites. Yet, the assignment of fish to the correct geographic region (Baltic region- North Sea- Irish/Celtic Sea) was higher (16 – 80 %). The low power of otolith shape in our study was in contrast to a study by Mérigot et al. (2007) that successfully applied shape morphometry to discriminate local sole populations in the Gulf of Lions, NW Mediterranean Sea. In that study an overall reclassification success of 62 % was obtained based on the combined use of Fourier coefficients and shape indices including a broad range of fish sizes and age classes. The discrimination between three local sites for only adult fish (1+) was even higher (94.5 %) (Mérigot et al. 2007).

Differences in growth rate are a prerequisite for otolith shape to be successful as a tool for population discrimination (Campana & Casselman 1993). For sole, differences in growth rate exist among the North Sea and Celtic Sea (Horwood 2001) and also between the North Sea and English Channel (Witthames et al. 1995). The higher discrimination power observed with otolith microchemistry compared to otolith shape might be explained because the otolith edge elemental signature refers only to the most recent environment of a fish, whereas otolith shape results from an integration of processes during the life history. It is possible that sole shared similar habitats or similar life conditions during parts of their life, homogenizing the spatial differences in otolith shape among sampling sites.

Conclusion

The present study demonstrates the use of otolith microchemistry and shape as a successful traceability tool to identify sole from wide geographical locations in the North-East Atlantic. In general, our results pointed to three large groups: Kattegat/Skagerrak, North Sea and Celtic/Irish Sea with distinct differences between groups and more subtle differences between samples within groups. The combined analysis of otolith microchemistry and otolith shape proved more powerful than either analysis in isolation for the samples from the North Sea, Celtic and Irish Sea. Although the temporal stability of this spatial signal should be investigated more carefully, these preliminary results are consistent with population genetic and tagging studies on *Solea solea* in the North Atlantic Ocean. The use of otolith microchemistry and shape to trace back the origin of landed fish at a moderate spatial scale will definitely help fisheries enforcement initiatives and could provide much needed complementary information about suspected fraud when genetics lacks small scale temporal resolution.

Acknowledgements

Our research was supported by the European Community's Seventh Framework Program under contract no. KBBE-213399 (FishPopTrace). The authors would like to thank E. Diopere, S. Verherstraeten, J. Guelinckx, K. Vancampenhout (KULEUVEN); U. Damm and N. Rohlf (ISH-BFAFI); E. Nielsen and colleagues (DIFRES); ILVO-Fisheries and the crew of RV Belgica and RV Zeeleeuw, for their help with the collection of the samples. Many thanks to J. Skadal (UiB), O. Tumyr and J. Kosler (Centre of Geobiology, UIB) for their help with the operation of the laser and ICPMS. We thank J. Guelinckx for comments and suggestions. We acknowledge the BeNCoRe network and Research Foundation Flanders for a travel grant. E.C. acknowledges a grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders. G.E.M. is a post-doctoral researcher funded by the Research Foundation Flanders.

SUPPLEMENTARY MATERIALS

Table S1 Standard Reference Material (SRM), mean and standard deviation of the limit of detection (LOD); percentage of data below LOD and relative standard deviation (% RSD) per element

	SRM	LOD	std LOD	% < LOD	% RSD
^{7}Li	612	0.473	0.924	37.3	15.1
^{23}Na	NIES	0.106	0.182	0.0	10.9
^{24}Mg	NIES	0.006	0.009	0.0	10.3
^{55}Mn	612	0.012	0.018	0.0	5.0
^{59}Co	612	0.014	0.012	11.5	5.2
^{63}Cu	NIES	0.003	0.003	13.4	24.1
^{66}Zn	NIES	0.006	0.007	16.8	26.7
^{85}Rb	612	0.001	0.002	18.2	5.8
^{86}Sr	NIES	0.051	0.077	0.0	8.8
^{111}Cd	NIES	0.000	0.000	1.6	24.9
^{138}Ba	NIES	0.001	0.002	0.0	15.2
^{208}Pb	NIES	0.000	0.000	37.2	33.5

Table S2 Element concentrations in $\mu g.g^{-1}$ (mean and standard deviation) measured in core (C) and edge (E). N = number of samples analysed. For sample codes, see Table 1

zone	Sample	Na			Mg			Mn			Co			Cu		
	Code	Mean	N	Std.Dev.	Mean	N	Std.Dev.	Mean	N	Std.Dev.	Mean	N	Std.Dev.	Mean	N	Std.Dev.
C	KAT07	2802.81	35	303.10	25.48	35	4.02	12.01	35	4.07	1.11	34	0.66	0.29	33	0.20
C	SKA07	2886.75	22	237.39	22.33	22	3.54	20.30	22	5.91	0.79	19	0.51	0.16	18	0.13
C	GER07	2629.46	34	265.38	27.53	32	6.74	16.85	34	9.77	1.18	32	0.66	0.19	29	0.09
C	BEL07	2584.14	26	291.23	24.73	26	3.89	11.56	26	2.86	1.63	26	0.75	0.06	26	0.04
C	BEL08	2730.87	30	216.94	20.94	30	4.96	14.27	30	6.32	0.69	25	0.50	0.14	24	0.09
C	THA07	2634.03	35	439.93	21.22	35	3.93	10.59	35	3.10	0.91	35	0.49	0.21	33	0.19
C	CEL08	2671.32	32	312.22	21.20	32	3.19	8.68	32	3.10	1.12	30	0.47	0.36	22	0.25
C	IS08	2691.72	29	241.67	21.94	29	3.28	13.81	29	9.66	0.67	24	0.42	0.16	27	0.14
E	KAT07	2175.20	35	267.77	16.14	35	3.25	7.27	35	1.97	0.66	32	0.45	0.18	29	0.10
E	SKA07	2319.29	22	265.18	12.72	22	2.33	15.35	22	4.48	0.65	20	0.47	0.10	18	0.09
E	GER07	2183.97	33	237.13	16.59	33	2.26	9.69	33	3.54	1.00	27	0.58	0.13	26	0.11
E	BEL07	2056.56	26	272.00	16.29	26	2.12	6.52	26	1.82	1.27	24	0.57	0.04	24	0.03
E	BEL08	2155.65	30	267.23	14.11	30	3.91	10.16	30	4.32	0.70	18	0.34	0.14	21	0.12
E	THA07	2279.24	35	312.46	16.17	35	2.32	7.62	35	1.37	0.65	33	0.45	0.12	30	0.12
E	CEL08	2185.16	31	215.64	13.87	31	1.30	5.68	31	1.04	0.70	26	0.44	0.19	28	0.14
E	IS08	2360.48	28	272.05	15.20	28	2.25	8.05	28	4.71	0.51	24	0.34	0.12	22	0.09
All		2457.20	483	381.09	19.23	481	5.65	10.91	483	6.14	0.81	482	0.63	0.14	478	0.15

zone	Sample	Zn			Rb			Sr			Cd			Ba		
	Code	Mean	N	Std.Dev.	Mean	N	Std.Dev.	Mean	N	Std.Dev.	Mean	N	Std.Dev.	Mean	N	Std.Dev.
C	KAT07	0.81	33	1.03	0.05	27	0.03	1950.90	35	218.51	0.005	35	0.003	7.25	31	3.98
C	SKA07	0.18	13	0.13	0.10	18	0.06	1942.89	22	252.55	0.001	20	0.002	5.46	21	3.89
C	GER07	0.68	30	0.75	0.04	28	0.04	2035.33	34	376.31	0.006	33	0.005	2.48	34	1.49
C	BEL07	0.11	22	0.09	0.06	23	0.05	1810.68	26	280.19	0.005	26	0.002	1.34	26	1.28
C	BEL08	0.72	24	1.03	0.08	24	0.08	1938.32	30	423.88	0.003	26	0.002	1.03	30	1.10
C	THA07	0.60	34	0.99	0.06	30	0.03	2062.48	35	336.57	0.007	35	0.005	1.43	35	0.89
C	CEL08	0.84	29	1.05	0.05	24	0.03	2058.28	32	375.45	0.004	32	0.003	3.00	30	3.15
C	IS08	0.59	27	0.54	0.03	19	0.04	2074.34	29	315.83	0.006	27	0.004	2.73	28	2.99
E	KAT07	0.58	25	1.11	0.04	28	0.03	2751.48	34	663.43	0.008	35	0.003	3.77	34	2.65
E	SKA07	0.22	18	0.23	0.10	18	0.08	2342.74	22	543.49	0.004	22	0.003	1.98	22	1.81
E	GER07	0.25	28	0.14	0.04	26	0.03	2117.58	33	423.10	0.011	33	0.009	1.34	33	0.84
E	BEL07	0.07	24	0.04	0.04	25	0.04	1973.66	26	383.26	0.007	26	0.002	0.56	26	0.20
E	BEL08	0.37	23	0.32	0.06	21	0.06	1942.45	30	412.62	0.006	30	0.002	0.79	30	0.53
E	THA07	0.26	29	0.33	0.08	33	0.11	2165.94	35	486.78	0.010	35	0.005	0.63	35	0.20
E	CEL08	0.36	20	0.35	0.03	27	0.02	2268.64	31	386.13	0.006	31	0.002	0.87	31	0.27
E	IS08	0.28	22	0.21	0.05	21	0.04	2153.62	28	427.60	0.008	28	0.004	0.78	28	0.49
All		0.40	482	0.69	0.05	483	0.06	2105.09	482	458.01	0.006	483	0.004	2.67	482	10.91

Chapter 4

Can otolith fingerprints help with tracing back the nursery origin of adult sole?

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ABSTRACT

In order to evaluate the relative importance of juvenile habitats, it is necessary to identify the juvenile habitats in which adult fish once lived. The chemical composition of juvenile sole (*Solea solea*) otoliths collected from four nursery grounds in the Southern Bight of the North Sea was compared to the chemical composition of the juvenile portion of adult otoliths collected from three main spawning grounds. Our results suggest a high level of recruitment to local populations for sole off the English Coast and higher connectivity off the continental European coast.

INTRODUCTION

Coastal areas and estuaries play an important role as nursery habitat for several marine fish and crustaceans (Beck et al. 2001; Able 2005). An area is considered a nursery habitat when it produces relatively more adults per unit of area than other juvenile habitats (Beck et al. 2001). One of the factors that need to be considered for evaluating the nursery role of habitats is the movement of individuals from nursery to adult habitats. Knowledge of the relative contribution of juvenile habitats to the adult fish stock is not only fundamental for understanding structure and dynamics of local populations; it also represents crucial information for sustainable management and the conservation of essential habitats (Beck et al. 2001).

Elemental fingerprints of otoliths have demonstrated their success in assessing connectivity between adult and juvenile populations and identifying natal origin of adult fish. Although most of these studies have been performed either on diadromous fish, estuarine spawning species or highly migratory species (Thorrold et al. 2001; Gillanders 2002a; Patterson et al. 2004; Rooker et al. 2008; Walther et al. 2008), more and more attempts are made to assess connectivity between the adult and juvenile habitats of marine spawning temperate fish species (Warner et al. 2005; Brown 2006; Vasconcelos 2007; Tobin et al. 2010; Wright et al. 2010).

One of the prerequisites for using otolith element composition as a natural tag is that it shows spatial differences between geographical locations. In an earlier study, we showed that the otolith fingerprints of juvenile sole (*Solea solea*) varied significantly among four nursery grounds in the North Sea (Chapter 2). Ten elements were measured with LA-ICPMS at the otolith edge and used in a forward stepwise Linear Discriminant Function Analysis. The juvenile sole collected in 2006 and 2007 were assigned to their respective nursery ground with a total cross-validated accuracy of 88 %. Six elements (Mn, Ba, Sr, Rb, Na, Mg) were important for this discrimination model for different nursery grounds. The question remains whether young sole recruit to the nearest spawning population and whether there are differences in nursery contribution. A more recent study on adults showed that three large spawning groups could be discriminated based on otolith edge fingerprints in the Northeast

Atlantic Ocean (Kattegat/Skagerrak- Irish Sea/Celtic Sea- North Sea) with distinct differences among groups and more subtle differences within groups (Chapter 3).

In this study, we analyzed the juvenile region of otoliths from adult sole collected at four important spawning grounds in the North Sea, in order to assess whether these adults could be assigned to their nursery ground of origin. The aim was to explore the relative proportions of adults originating from different nursery grounds in order to define the ecologically important nurseries on the basis of their contributions to the adult populations.

MATERIALS AND METHODS

Sampling

Juvenile sole were collected off the Thames (THA), off the Humber (LINC), in the Wadden Sea near Texel (TEX) and in the Scheldt estuary near Zandvliet (ZAN) (for more details, see Chapter 2). Adult sole were collected during research surveys in 2007, at three spawning grounds in the North Sea: off the Thames, along the Belgian Coast and in the German Bight (Fig. 1). The sample from the English Coast (THA) was collected outside the spawning period; all other samples were collected in the spawning season (Table 1). The Belgian spawning population was also sampled in 2008, to assess interannual variation. Sagittal otoliths were extracted onboard or in the lab and kept in trays or eppendorf vials. From each location, about 20 - 30 otoliths were selected for otolith microchemistry, resulting in a total of 125 adult sole otoliths. Fish of similar length were chosen in order to minimize the effects of any size-specific trends in otolith composition. Left sagittae were used for the microchemistry analysis, except in a few cases when only the right sagitta could be retrieved.

Table 1 Sampling information on adults of *Solea solea*. Sample code and location, sampling year (* indicate spawning sample) and month, coordinates of sampling location, number of otoliths (N), total fish length (TL) (mean ± standard deviation) and otolith weight (OW) (mean ± standard deviation).

Sample code	Location	Year	Month	Latitude	Longitude	N	TL (cm)	OW (mg)
GER07	German Bight	2007*	May	54°31'12"N	7°53'23"E	34	25.43 ± 4.45	10.43 ± 4.53
BEL07	Belgian Coast	2007*	May	51°21'16"N	2°56'11"E	26	26.29 ± 3.86	11.57 ± 4.43
BEL08	Belgian Coast	2008*	May	51°23'22"N	3°10'01"E	30	26.98 ± 0.73	13.15 ± 2.63
THA07	Thames	2007	August	51°27'80"N	1°20'00"E	35	26.77 ± 1.57	13.37 ± 3.32

Otolith microchemistry

Details of the sample preparation are described in Chapter 3. Laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) analyses were carried out using a Finnigan ELEMENT 2 ICP-MS (Thermo Electron Corporation, Bremen) coupled to a UP266 MACRO laser system (New Wave Research). Laser conditions (10 Hz repetition rate, 50 ms dwell time, medium resolution mode) induced a crater of 60 µm diameter at a distance of 150 µm - 200 µm from the core, covering the juvenile portion of the adult otolith (referred to as 'Ad_j'). Previous studies on sole have shown that this area corresponds to material deposited during the juvenile growth season after metamorphosis and before the formation of the first annulus (Lagardère & Troadec 1997, de Pontual et al. 2003). The following thirteen isotopes were measured: ^{7}Li, ^{23}Na, ^{24}Mg, ^{43}Ca, ^{55}Mn, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{85}Rb, ^{86}Sr, ^{111}Cd, ^{138}Ba and ^{208}Pb. Counts per second were processed using the software GLITTER (GEMOC, Macquarie University) with ^{43}Ca selected as internal standard. The calibration of the instrument was performed using certified glass reference material (NIST610) (Supplementary Materials, Table S1). Standard reference materials (NIST610, NIST612, NIES) were measured every other 15 spots. The minimum detection limit at the 99 % confidence level was calculated by GLITTER using an algorithm developed by Longerich et al. (1996). Data below the limits of detection were set to the limit of detection, as described in chapter 2.

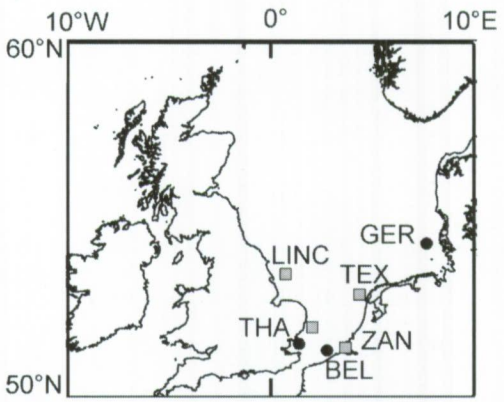


Fig. 1 Sampling locations for *Solea solea*. Black circles indicate adult sampling locations and grey boxes indicate juvenile sampling locations.

Data analysis

Data from adult sole (Ad_j) were combined with the data from juvenile sole (edge fingerprints; referred to as 'J') described in Chapter 2. The concentrations of Na, Mg, Mn, Cu, Zn, Rb, Sr and Ba were log_{10} transformed and Co square root transformed, to meet requirements for data normality and homogeneity of variance. Li and Pb were removed from the dataset because more than 30 % of the data fell below the detection limit in the adult sole otoliths (Supplementary Materials, Table S1). Also Cd was removed because this element was not included in the juvenile baselines. This resulted in a final dataset with nine elements shared with the study of Chapter 2.

Differences in mean element concentrations measured in juvenile otoliths (J) and in the juvenile area of the adult fish ('Ad_j') were analyzed with a t-test for independent samples. For this test, all samples from the different locations were pooled. Further, a principal component analysis (PCA) was performed to visualize if adult 'nursery data' fell within the same multidimensional space as the juvenile data. These tests were necessary because the year classes of the baseline data (juveniles) did not match the year classes of the adults and because not all potential coastal nursery areas that could have contributed to the adult fish were sampled. Signatures that were outside the juvenile space on the PCA plot could possibly have originated from non-sampled areas.

Next, to test for spatial differences in element concentration of the nursery signal of adult sole among sampling locations, multivariate and univariate ANOVA were used.

Finally, we used the direct maximum likelihood estimator (MLE) implemented in the program HISEA (Millar 1990b) to determine the proportion of adults that are assigned to each one of the four nursery grounds (i.e. juvenile baseline data). Maximum likelihood estimators are expected to provide more discrimination power than classification-based estimators in mixed stock situations (Millar 1990a). We used the bootstrap mode of the program, with 1000 simulations. To account for the interannual variability observed between the two temporal samples from the Scheldt nursery (chapter 2), the data from 2006 and 2007 were pooled as one baseline.

RESULTS AND DISCUSSION

The concentration of nine elements (Na, Mg, Mn, Co, Cu, Zn, Rb, Sr and Ba) was compared between juvenile and adult sole otoliths. Despite the comparison of adult sole from a different year class to the juveniles composing the baselines, the element concentrations measured in the nursery portion of adult otoliths (Ad_j) fell within the range measured in the juvenile sole otoliths (Fig. 2). Nevertheless, the average concentrations differed for seven elements (Na: $t = -3.66$, $p = 0.0002$; Mg: $t = -5.10$, $p < 0.0001$; Mn: $t = -3.05$, $p = 0.002$; Co: $t = -2.08$, $p = 0.04$; Cu: $t = -2.08$, $p = 0.04$; Rb: $t = 7.74$, $p < 0.0001$; Sr: $t = -5.45$, $p < 0.0001$).

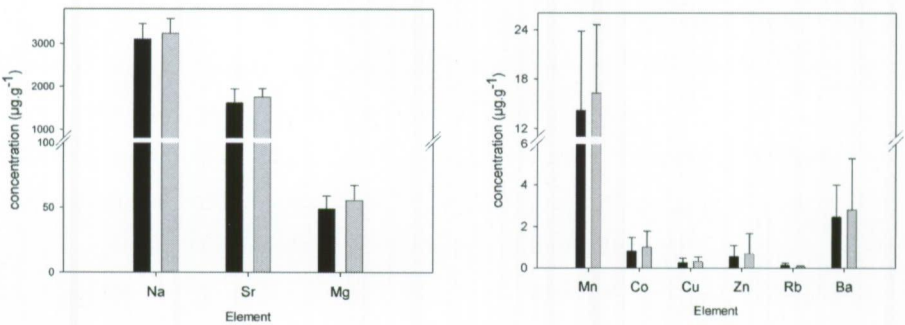
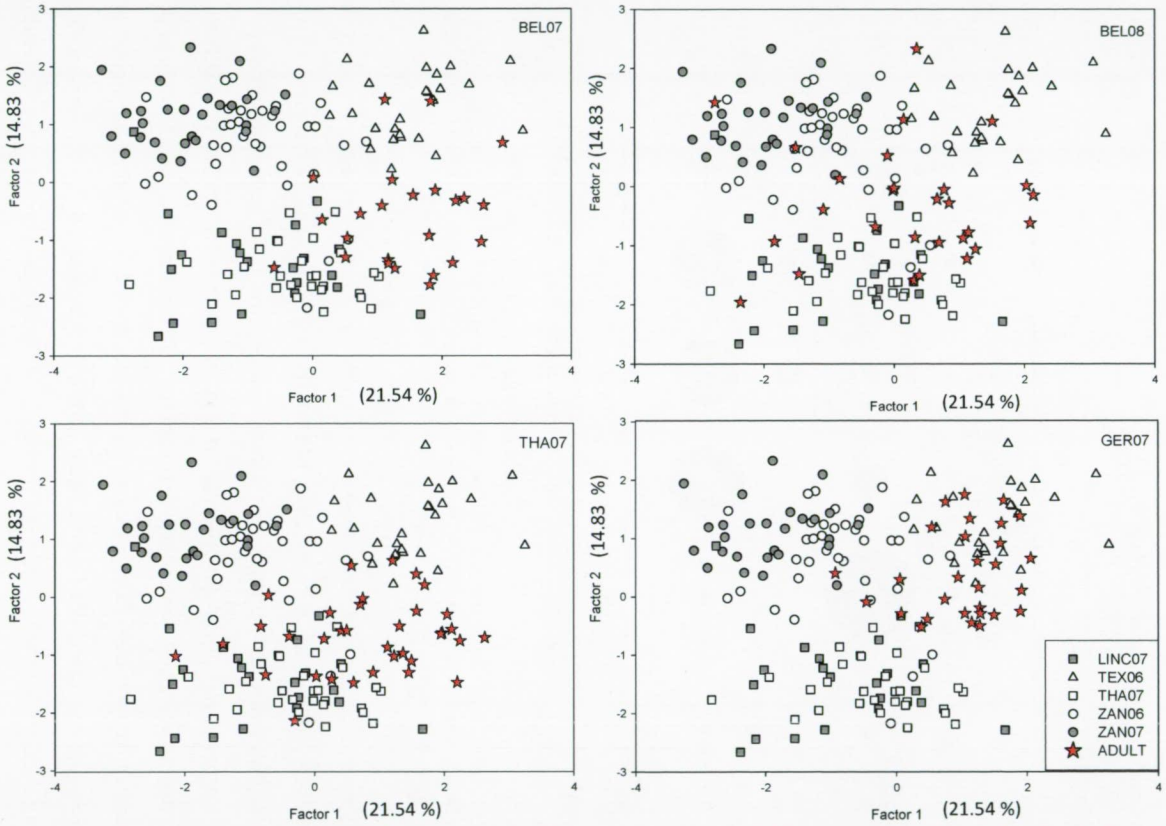


Fig. 2 Mean concentrations ($\mu g.g^{-1}$) of elements in samples of juvenile otoliths (black bars-'J') and in the nursery portion of adult otoliths (grey bars-'Ad_j') (All locations pooled). Values represent Mean and Standard Deviation.

When we compared the multi-element fingerprints of the baseline data (juvenile sole) to that of the mixed data (adult sole) on the PCA plot, it was apparent that most of the adult sole occupied the space defined by the juvenile groups (Fig. 3). However, some of the adults caught off the Thames and along the Belgian Coast (BEL07) did not cluster with any of the juvenile groups. Possibly, these individuals originated from nursery areas that were not sampled or, alternatively, this could be the result of the temporal mismatch of juveniles and adults. Ideally, adults should come from the same year class as the juveniles because otolith elemental composition might fluctuate annually between environments (Gillanders 2002b).



	Factor 1	Factor 2
Na	0.763	-0.105
Mg	0.841	0.142
Mn	0.564	0.535
Co	0.338	-0.287
Cu	0.084	0.137
Zn	0.269	0.196
Rb	-0.079	0.101
Sr	0.336	-0.612
Ba	-0.136	0.702

Fig.3 Principal Component Analysis of juvenile fingerprints and adult 'nursery' fingerprints. Adult samples from different locations (indicated in upper right corner of each graph) are indicated with a star. Factor loadings are presented. For sample codes of adults, see Table 1. Grey and white symbols represent juvenile samples. Sample codes of juveniles: TEX= Texel, LINC= Lincolnshire coast, THA= offshore Thames, ZAN= Scheldt estuary; last digits indicate sampling year.

Significant differences in multi-element fingerprint of the juvenile region (Ad_j) were present among the adult sole sampled at different spawning grounds (MANOVA; $F_{63,1098} = 6.5$, $p < 0.0001$). Otolith composition differed significantly for five elements (ANOVA, df = 7,202: Na: $F = 8.87$, $p < 0.0001$; Mg: $F = 9.21$, $p < 0.0001$; Mn: $F = 14.02$, $p < 0.0001$; Co: $F = 3.38$, $p = 0.001$ and Ba: $F = 26.09$, $p < 0.0001$). This suggests that adults caught at the various locations are characterized by a different nursery origin, which was confirmed by the results of the maximum likelihood analysis (Fig. 4).

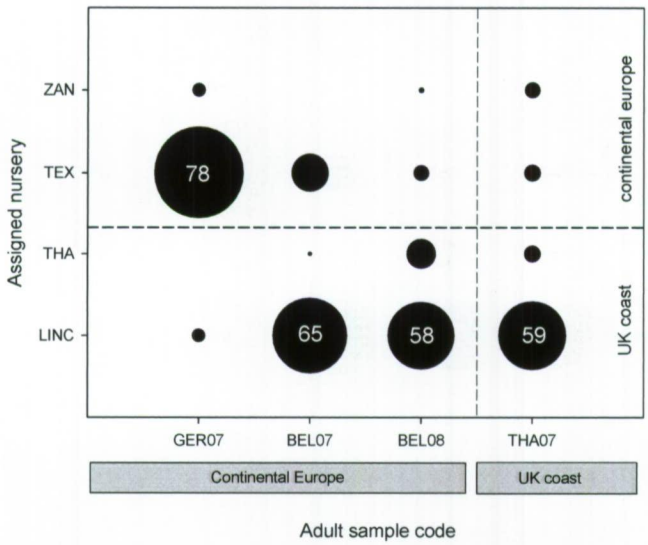


Fig. 4 Direct maximum likelihood estimates of relative contribution of juvenile baselines to adult populations of *Solea solea*. Sample codes of juveniles: TEX = Texel, LINC = Lincolnshire coast, THA = offshore Thames, ZAN = Scheldt estuary; for more details see Chapter 2. For sample codes of adults, see Table 1.

The adults caught off the Thames showed only a small proportion of assignment to the Thames nursery (14 %), while more than half of the fish (59 %) were assigned to the northeast English Coast (LINC). This is not unexpected because the discrimination power of juvenile fingerprints was higher on opposite sides of the southern North Sea than between samples collected along the same coastline (Chapter 2). This means that in total, 73 % of the fish sampled off the Thames were allocated to the nurseries off the UK coast, suggesting local recruitment and some site fidelity. Most of the adults sampled in the German Bight (78 %) were allocated to the Wadden Sea nursery (TEX). Northward movement of sole from

the Dutch coast into the German Bight has been observed in physical tagging studies as well (Burt & Millner 2008). Comparing the signatures of the spawning population from the Belgian Coast with the juvenile signatures, it seemed that several nursery areas contributed to this spawning population, with variation in the relative contribution of the different nurseries between both years. It was not the closest nursery of the Scheldt estuary that showed the highest contribution to this population, but the Northeast English Coast (LINC) in both years. Other nurseries showed also small contributions to this spawning population (Fig. 4). The high proportion of adults assigned to English nursery grounds for sole spawning along the Belgian coast contrasts with tagging studies, namely that juvenile sole tagged along the continental coast do not substantially recruit to the English Coast populations and vice versa (Rijnsdorp et al. 1992). The percentage of fish recaptured along the UK coast was about 2-10 % in tagging studies and much higher in our study. Our estimates will likely present an overestimate of the sampled nursery areas because not all possible sources are included. Besides the presence of spatial variation in chemical composition, other assumptions have to be fulfilled in order to use otolith microchemistry for studying connectivity between juvenile and adult fish (Campana et al. 2000). All possible groups contributing to the mixture should be characterized. For sole, this is nearly impossible because the nursery grounds form an almost continuous zone along the continental shorelines and the English Coast (Rijnsdorp et al. 1992). Nevertheless, the four main nursery areas in the North Sea are identified as the Eastern UK coast, the German Bight, the Wadden Sea (Southern Bight) and the Belgian Coast and Scheldt (Rijnsdorp et al. 1992; Horwood 2001). In future studies, juveniles from the Belgian coastal area and from the German Bight should be included. Local effects may play a fairly important role. For example, the chemical signal from the Scheldt might differ from that of the Flemish Banks (Belgian coast), at a distance of only 50 km, because of temporal variation in salinity, fresh water runoff and temperature in the Scheldt (Lacroix et al. 2004; Van Damme et al. 2005).

Based on this explorative study and the results described in chapter 2, it seems that the movement of juvenile sole is limited, enabling them to build up a chemical signal from the nursery ground where they grow up. Later on, sole possibly recruit to one of the local spawning populations. The adult samples in our study were composed of mixed ages and so the technique should be robust enough to find patterns that are stable over several years.

Nevertheless a more adequate spatial and temporal sampling design is required to confirm the hypothesis of local recruitment and to evaluate the nursery contribution and relative value of these nursery grounds.

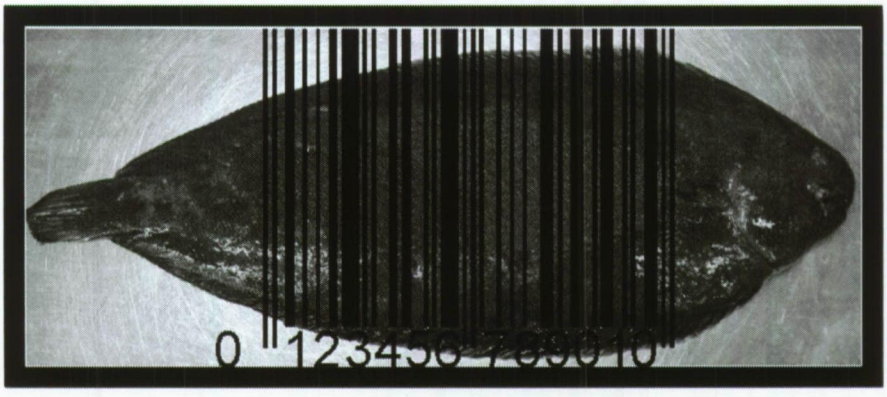
Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement n° KBBE-212399 (FishPopTrace). The authors thank S. Geldof and J. Guelinckx (K.U.LEUVEN), U. Damm and N. Rohlf (ISH-BFAFI), E. Nielsen and colleagues (DIFRES), ILVO-Fisheries and the crew of RV Belgica and RV Zeeleeuw, for help with the collection of samples. Many thanks to J. Skadal (UIB), O. Tumyr and J. Kosler (Centre of Geobiology, UIB) for help with the operation of the laser and ICP-MS. Acknowledgements to BeNCoRe network and Research Foundation Flanders (FWO Vlaanderen) for a travel grant. E.C. acknowledges a grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). G.E.M. is a post-doctoral researcher funded by the Research Foundation Flanders (FWO Vlaanderen).

SUPPLEMENTARY MATERIALS

Table S1 Mean limit of detection (LOD), percentage of data below LOD and relative standard deviation (RSD) of all elements measured in the juvenile portion of adult otoliths.

	LOD	% < LOD	% RSD
Li	0.081	50.8	10.2
Na	0.110	0.0	8.4
Mg	0.015	0.0	3.4
Mn	0.012	0.0	5.8
Co	0.016	12.2	10.4
Cu	0.007	10.5	12.6
Zn	0.016	17.3	9.8
Rb	0.002	14.9	9.1
Sr	0.044	0.0	2.9
Cd	0.014	0.8	9.9
Ba	0.001	0.0	2.9
Pb	0.001	39.4	9.5



Chapter 5

The power of integrating genetics and otolith microchemistry into the management of exploited marine fishes: the case of sole (*Solea solea*) in the North-East Atlantic.

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ABSTRACT

Marine populations exhibit various degrees of population discreteness, ranging from an historical evolutionary till contemporary ecological level of differentiation. An integrated approach combining complementary population discrimination tools can provide more powerful results to study the level of connectivity between populations and to increase the traceability power in management applications. Here, we combined the results obtained from ten microsatellite markers with results from otolith microchemistry analyses on adult sole (*Solea solea*) populations in the North-East Atlantic Ocean. Major large scale differentiation was detected between three distinct regions (Baltic transition area, North Sea, Irish/Celtic Seas) with both types of markers. At the within regions scale, genetic differentiation lacked power for reliable assignments of individuals to sampling locations, while otolith microchemistry provided more resolution power. An integrated discriminant model combining genetic data with microchemistry data further improved the traceability power for various locations. Our results highlight the power gained by a multi-marker approach depending on the required resolution scale, enabling the use of even subtle variations between populations for traceability purposes.

INTRODUCTION

Many marine fish are characterized by a high fecundity and larval forms with a long pelagic duration, increasing the likelihood of large dispersal ranges. Nevertheless, several studies recently suggested that local retention of larvae and self-recruitment might be higher than expected (Jones et al. 1999; Sponaugle et al. 2002; Swearer et al. 2002) and that marine populations more likely consist of a network of connected populations within a metapopulation (Thorrold et al. 2001; Wright et al. 2006). Furthermore, the unexpected genetic structure found in some marine populations (Knutsen et al. 2003; Nielsen et al. 2004) and evidence for larval behavior (Cowen et al. 2000; Grioche et al. 2001; Bradbury et al. 2003) question the generally assumed 'open models'. To fully understand the dynamics of marine populations, the integration of biological information, genetic information and physical oceanography can be successful (Gilg & Hilbish 2003; Galindo et al. 2010; White et al. 2010).

Multiple methodologies are available for studying population structure in marine fish: genetic markers (Hauser & Carvalho 2008), otolith microchemistry (Campana et al. 1994; Jonsdottir et al. 2006b) and otolith shape (Campana & Casselman 1993; Jonsdottir et al. 2006a), parasite load (Charters et al. 2010) and morphometrics (Turan 2004) have all shown their usefulness to discriminate between populations. Genetic markers can identify reproductively isolated groups which represent the most relevant eco-evolutionary units for management strategies (Palsbøll et al. 2007; Reiss et al. 2009). For example microsatellite loci can assign marine fish to their capture location, even in taxa that are generally known for their low levels of genetic differentiation (Nielsen et al. 2001; Ruzzante et al. 2006).

Spatial variation in other natural tags may indicate additional ecologically or demographically independent groups of individuals and help to address connectivity questions. The chemical composition of the otolith core provides an environmental record from the period shortly after spawning; differences in core chemistry might reflect spatial segregation during spawning (Ashford et al. 2006). Otolith core chemistry can thus be used in complement to genotypes to address population structure. The otolith edge on the other hand provides a record of the capture location and can be used for traceability purposes. Traceability tools aim at tracking landed fish or fish products back to its origin of capture and

are necessary for consumer protection and for controlling illegal, unreported and unregulated fishing (Ogden 2008).

Genetic data and otolith microchemistry have often been used separately, but recent studies combining markers have shown that the integration of multiple techniques may potentially increase the power for detecting population structure and traceability applications. For instance, the combined analysis of genetic markers and otolith microchemistry revealed that results can either be corroborative (Patterson et al. 2004; Miller et al. 2005; Bradbury et al. 2008; Miller et al. 2010), complementary (Ashford et al. 2006; Feyrer et al. 2007; Higgins et al. 2010) or conflicting (Thorrold et al. 2001).

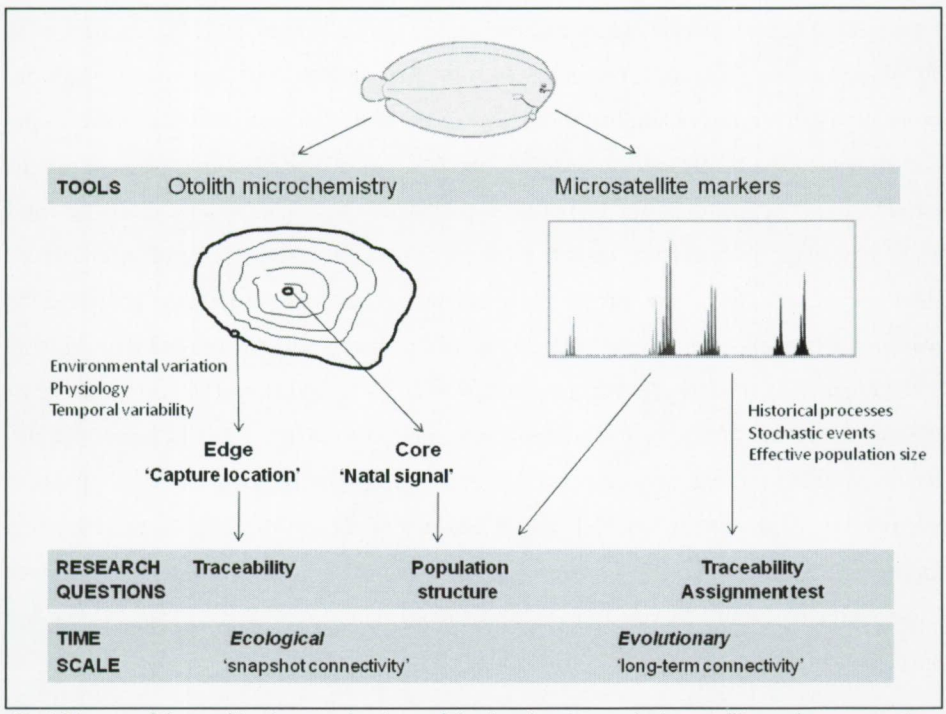

Fig. 1 Diagram of the combined approach using otolith microchemistry and microsatellite markers, presenting the research questions that can be tackled with each tool and the time scale of study for both types of markers.

Integrating genetic and ecological markers provides a challenge, because they give an estimate of connectivity at different time scales. While genetic markers provide long-term estimates of population structure, otolith microchemistry provides a short-term estimate of connectivity. Chances for obtaining a high resolution power for both microchemical and

genetic markers are limited, especially in marine fish (Selkoe et al. 2008). The use of otolith microchemistry as a population discrimination tool requires environmental variation and temporal stability of the signal, while genetic markers are strongly influenced by historical and stochastic events and by effective population size (Fig. 1). The combination of different markers makes it therefore possible to obtain complementary information and to get higher validation and precision because each marker type displays different types of limitation and performance (Fromentin et al. 2009).

Sole (*Solea solea* L.), a commercial flatfish living in the coastal waters of the North-East Atlantic Ocean and Mediterranean Sea, shows low genetic differentiation among Atlantic populations, following an isolation-by-distance model, and larger vicariant differences between the Atlantic Ocean and Mediterranean Sea (Kotoulas et al. 1995; Exadactylos et al. 1998; Exadactylos et al. 2003; Rolland et al. 2007; Maes & Volckaert pers.comm.). A recent population genetic study identified three large groups based on microsatellite markers- 'the Baltic region', 'the North Sea and Irish Sea' and 'the Bay of Biscay'- with significant genetic differences between groups and more homogeneity among samples within clusters (Chapter 1). Otolith shape analysis could identify different sole populations in the Mediterranean Sea (Mérigot et al. 2007) and otolith microchemistry has been successfully applied to discriminate among juvenile sole caught in various nurseries in the North Sea (Leakey et al. 2009, Chapter 2), the Bay of Biscay (de Pontual et al. 2000) and along the Portuguese coast (Vasconcelos et al. 2008). Limited juvenile movement of sole in the Bay of Biscay has also been confirmed through parasites (Durieux et al. 2010). The combined analysis of otolith microchemistry and shape resulted in high assignment success of adults to the collection locations in the North-East Atlantic (Chapter 3). Nevertheless, none of these studies have assessed the power of integrated genetic and ecological marker data to investigate large and small scale population structure for traceability purposes.

The general aim of the present study was to improve the understanding of the connectivity of *Solea solea* in the North-East Atlantic Ocean. We used genetic markers (microsatellite DNA) and otolith microchemistry to investigate population structure. Because the otolith core may record spatial segregation during spawning, results from the otolith core microchemistry (Chapter 3) were compared with microsatellite genotypes (Chapter 1) obtained from the same adult sole, and further integrated in a joint analysis.

We also tested if the traceability to the capture location could be improved by the combined use of genotypes and microchemistry of the otolith edge.

MATERIALS AND METHODS

Sample collection

In total 535 adult sole were collected during research surveys in 2007 and 2008, at seven spawning grounds in the Southern Bight of the North Sea, the Celtic and Irish Sea and in the Skagerrak and Kattegat (Fig. 2, Table 1). Fish were either immediately stored frozen or measurements were done onboard and a fin tissue sample was preserved in 96 % ethanol for genetic purposes. Sagittal otoliths were extracted onboard or in the lab and kept in trays or eppendorf vials. From each location, about 30 - 40 otoliths were selected for otolith microchemistry and shape, resulting in a total of 214 otoliths. Fish of similar length were chosen in order to minimize any size effects. Left sagittae were used for the microchemistry analysis, except when only the right sagitta could be retrieved.

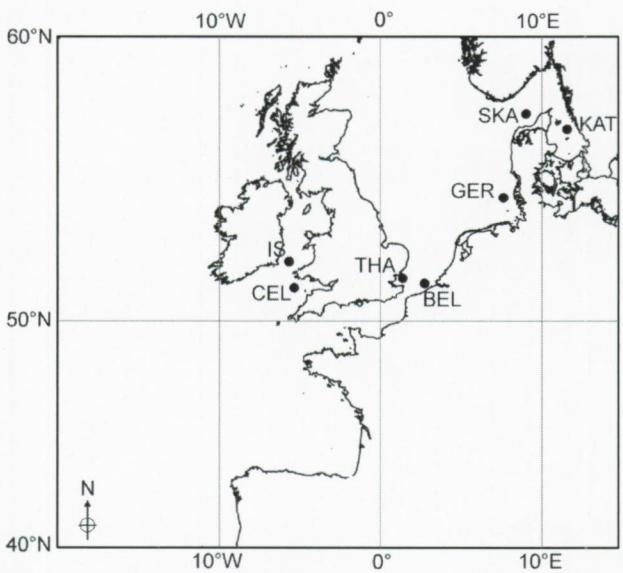

Fig. 2 *Solea solea*. Map with sampling sites. For sample codes, see Table 1

Table 1 Sampling information for *Solea solea*. Sample code, reference to Fig. 2, location, sampling year (* indicate spawning samples) and month, latitude and longitude, number of samples (N), number genotyped (Ngeno), number analyzed for otolith microchemistry (N mc) and numbers in common (Ngeno&mc)

Sample code	Map	Location	Year	Month	Latitude	Longitude	N	N geno	N mc	N geno&mc
KAT07	KAT	Kattegat	2007	Nov	57°08'91"N	11°38'52"E	48	44	35	32
SKA07	SKA	Skagerrak	2007	Nov	58°09'43"N	9°30'32"E	48	39	22	19
GER07	GER	German Bight	2007*	May	54°31'12"N	7°53'23"E	60	53	34	29
BEL07	BEL	Belgian Coast	2007*	May	51°21'16"N	2°56'11"E	96	96	26	13
THA07	THA	off Thames	2007	Aug	51°27'80"N	1°20'00"E	96	94	35	17
CEL08	CEL	Celtic Sea	2008*	Apr	50°49'00"N	5°01'00"W	96	81	32	30
IS08	IS	Irish Sea	2008*	Mar	52°13'00"N	5°20'00"W	91	91	30	28
Total							535	498	214	168

Microsatellite data

Details on the DNA extraction and genotyping protocol are presented in Chapter 1. The microsatellite dataset used in this study consisted of 498 adult sole, genotyped for ten microsatellite loci. To evaluate the allocation success of individuals to their sampling site based on the multilocus genotypes, we compared two different approaches. First, we used the frequency based method of Banks and Eichert (2000), implemented in the software WHICHRUN v.3.2. To determine individual assignment, the jackknife procedure was used, in which each individual was sequentially removed and treated as unknown. Individuals are then assigned to the baseline sample with the highest likelihood. Second, we applied the method of Rannala & Mountain (1997) implemented in the software GENECLASS v. 2.0 (Piry et al. 2004) to calculate average classification success to each population. The latter method differs from the previous in that it uses a Bayesian approach to estimate the population allele frequencies from the sample allele frequencies, while WHICHRUN assumes that the sample allele frequencies represent the population accurately (Pearse & Crandall 2004). These analyses were done on the maximum number of genotyped individuals (N = 498) in order to maximize the discrimination power.

Otolith microchemistry data

In total, 214 fish were used for otolith microchemistry; the number analyzed with otolith microchemistry is lower than the number analysed with microsatellite markers due to the sufficient power of the technique with lower sampling size (30-40 individuals) and the higher cost per sample compared to microsatellites. For details on otolith preparation and laser-ablation ICP-MS we refer to Chapter 3. The otolith microchemistry dataset consisted of concentrations ($\mu g.g^{-1}$) of ten elements (Na, Mg, Mn, Co, Cu, Zn, Rb, Sr, Cd, Ba), measured at the otolith core (C) and the otolith edge (E). Spatial variation in otolith edge and core was observed in an earlier study using multivariate statistics. Discriminant models based on edge microchemistry have been successfully applied to predict collection site with an overall assignment success of 55 % (Chapter 3).

Data analysis

We initially compared independently the results of the linear discriminant function analysis (LDFA) from the previous study to the assignment success based on microsatellite genotypes as explained above. In order to compare results from both markers from the same individuals, we applied a linear discriminant function analysis on the reduced dataset, containing only those individuals that were also genotyped (see Table 1 for sample sizes).

We used the core microchemistry data to investigate population structure and the edge microchemistry data to evaluate the traceability power.

In an attempt to integrate more efficiently all available information from both types of markers (genetic data and microchemistry data) into a combined approach to study population structure and traceability, we first calculated the correlation between two dissimilarity matrices with a Mantel test (Mantel 1967) in the Vegan library in R (Oksanen et al. 2007). The first matrix was based on genetic distance (Rousset 1997) and the second on Euclidean distance among samples, calculated from the average standardized element concentrations measured in the otolith core, representing the spawning signal (Mantel test 1) and otolith edge, representing the capture location (Mantel test 2), respectively. The statistical significance was tested using 10 000 permutations. Then, the two-dimensional scaling (MDS) ordinations of the two types of markers (genetic and otolith microchemistry) were compared by a Procrustes Analysis (Gower 1975) using R software. This method searches for the best match between two configurations of points in a multivariate Euclidean space using rotation, reflection, translation and dilation of one configuration. The minimization of the sums of squares between the differences for each observation (m^2) is used as the criterion to assess the best fit. The significance of the result and optimal superposition of one configuration on the other is obtained through a permutation test (PROTEST, (Jackson 1995)). This test is using R = sqrt ($1-m^2$) as a test statistic which can be interpreted as a correlation.

To evaluate if the assignment success could be improved by combining genetic data with microchemistry data into a single analysis, we developed a linear discriminant model using input variables from both types of markers. Because the genetic data are not continuous variables that can be entered directly in a discriminant model, we first performed a factorial correspondence analysis (FCA) based on the multilocus genotypes of the reduced dataset in GENETIX v.4.05 (Belkhir et al. 2004). The FCA, displaying the individuals in a multidimensional

hyperspace, was done with the option '3D' without prior information on populations. Next, we performed a principal component analysis (PCA) on the core microchemistry data and on the edge microchemistry data, respectively, and combined the first 10 principal component factors resulting from this analysis with the first 10 axes from the FCA analysis to estimate the cross-validated classification success. The combined model using the *core* microchemistry data together with genotypic information was used to compare results on *population structure*. The combined model using the *edge* microchemistry data together with genotypic information was used to compare results on *traceability*. The cross-validated assignment success from these two combined analyses was compared to the assignment success from the reduced dataset, based on either type of markers separately. The assignment success based on genetic markers was calculated from the correctly assigned individuals in WHICHRUN.

RESULTS

The assignment success of individuals to their collection location was much higher based on otolith edge microchemistry compared to the genetic assignments at all sampling locations, except for the Irish Sea (Table 2).

Table 2 Percentage successful assignment to sampling location based on microsatellite genotypes using GENECLASS (Piry et al. 2004) and using WHICHRUN (Banks & Eichert 2000), and cross-validated assignment scores from the linear discriminant analysis based on otolith microchemistry edge (MC (E)) and otolith microchemistry core (MC (C)). Analyses are done on the maximal number of samples possible.

		Assignment succes (%)			
		Geneclass	Whichrun	MC (E)	MC (C)
Sample code	KAT07	16.0	25.0	88.2	74.3
	SKA07	10.0	41.0	68.2	72.7
	GER07	25.0	33.3	36.4	53.1
	THA07	19.0	23.4	44.1	45.7
	BEL07	28.0	36.4	69.2	80.8
	CEL08	22.0	41.8	71.0	46.4
	IS08	19.0	56.5	28.6	31.0

Moreover, the performance of both genetic assignment methods differed strongly. The classification success based on the ten microsatellite markers in GENECLASS varied from 10 % for the Skagerrak sample (SKA07) to 28 % for the Belgian spawning population (BEL07). The

assignment success in WHICHRUN was higher for all samples and varied from 23 % for the Thames sample (THA07) up to 57 % for the Irish Sea (IS08). The results of the latter assignment test also showed that a total of 61 % of the Kattegat samples and 67 % of the Skagerrak individuals were assigned to one of the samples from the Baltic region (SKA or KAT) (Fig. 3). The Irish Sea and Celtic Sea showed moderate to high self-assignment scores, suggesting a spawning population that is genetically distinct (Table 2). Nevertheless, the assignment power to the main sampling region ('Baltic region', 'North Sea', 'Celtic/Irish Sea') was more than 74 % for all three regions based on otolith edge microchemistry, while this percentage varied between 42 % and 67 % based on microsatellite markers (Fig. 3).

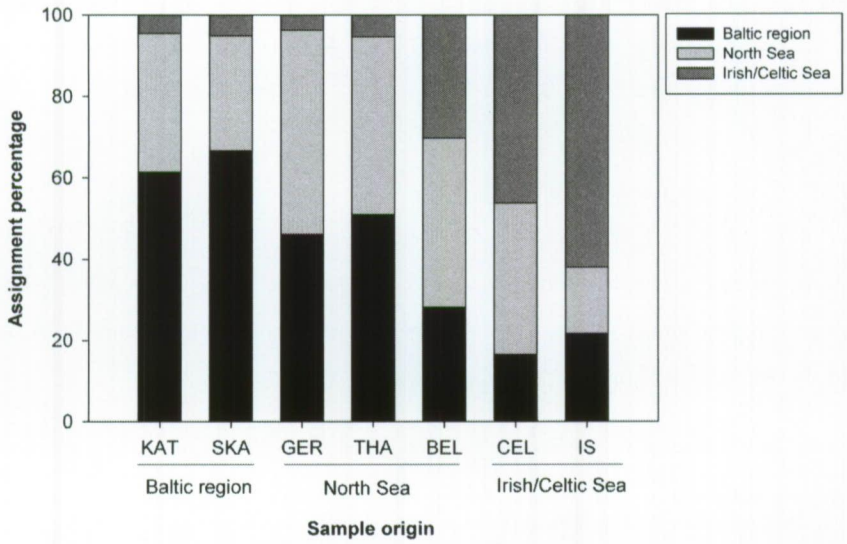

Fig.3 *Solea solea*. Assignment percentages to the three regions (coloured bars) based on genotypes at 10 microsatellite loci, calculated with the method of Banks and Eichert (2000). For sample codes, see Table 1.

The Mantel test indicated a significant positive correlation between genetic distance and Euclidean distance of the core microchemistry data (Mantel 1: $R = 0.483$, $p = 0.013$) and between genetic distance and Euclidean distance of the edge microchemistry data (Mantel 2: $R = 0.444$, $p = 0.008$). The graphical presentation of the correlation between the two data types shows that even for low genetic differentiation ($F_{ST} < 0.002$), the Euclidean distance calculated from the microchemistry data was sometimes high (Fig. 4). For instance, the samples 'Kattegat-Skagerrak', 'Skagerrak-German Bight' and 'Belgian Coast-Irish Sea', all had a pairwise $F_{ST} < 0.002$ while the Euclidean distance based on the core microchemistry was

higher than 2.1. Also the edge microchemistry signal between the 'Kattegat-Skagerrak' and 'Skagerrak-German Bight' showed large differences, while they were genetically very similar (Fig. 4).

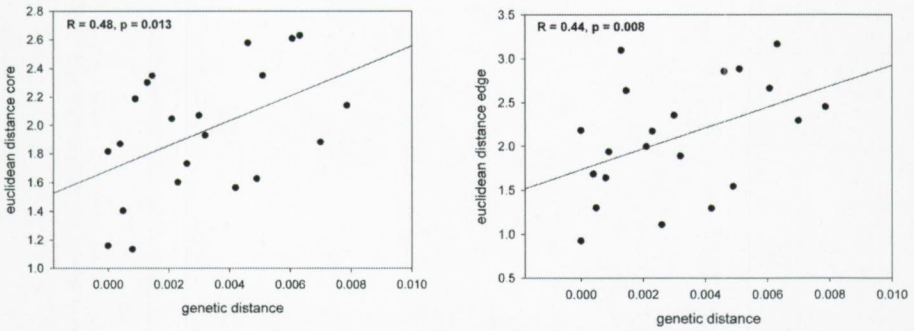

Fig. 4 *Solea solea.* Correlation between genetic distance (pairwise F_{ST} Rousset 1995) and Euclidean distance from microchemistry data of otolith core (left panel) and edge (right panel).

Two-dimensional representation of the seven samples illustrated that, based on the core microchemistry, the samples from Skagerrak were the most distinct. The samples from the Belgian Coast and the German Bight were also different from all other samples. Based on genetic differentiation among samples, the Belgian population resembles more the Thames and Celtic Sea and the German Bight presents the transition between Baltic region and the remaining populations (see Supplementary Materials, Fig. S1). The graphical ordination of the Procrustes Analysis was not significant ('core vs F_{ST}': R = 0.533, p = 0.291; 'edge vs F_{ST}': R = 0.629, p = 0.113) suggesting that it was impossible to significantly match the conformation of the MDS plot based on the genetic distance with the MDS plot based on otolith differences (Fig. 5). The information from both markers is thus not completely redundant.

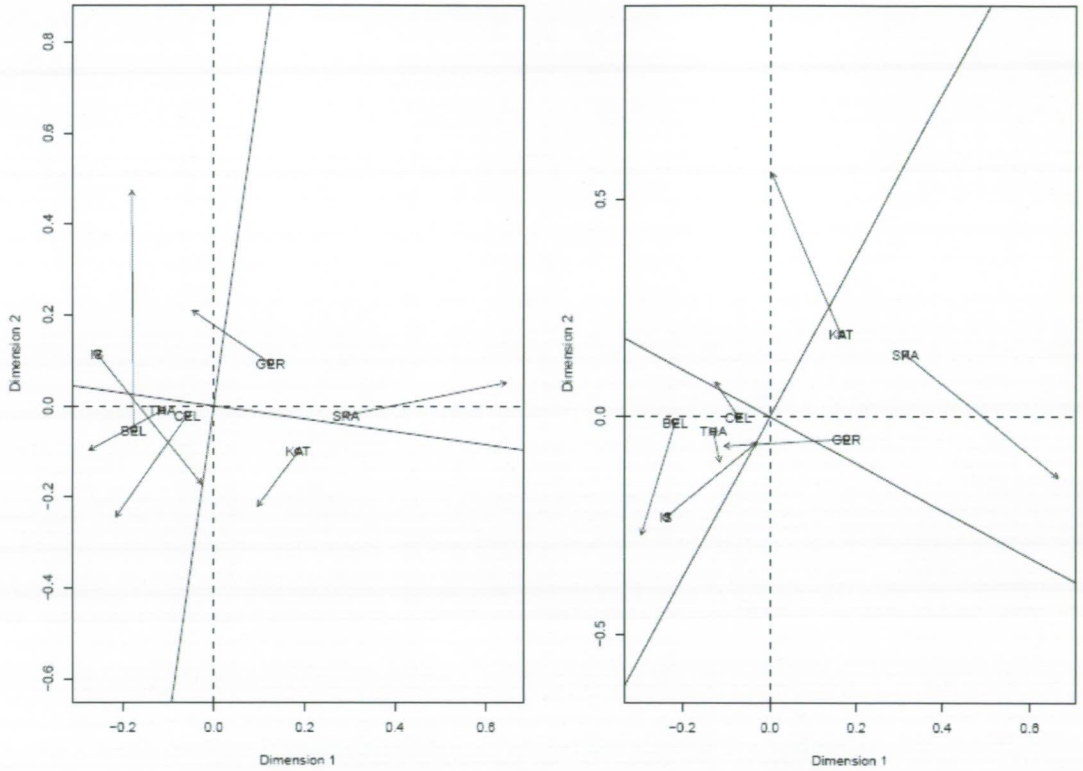

Fig. 5 Procrustes configurations. Codes represent samples used in data analysis (see Table 1). Best-fit configuration of matrix based on Euclidean distance of core microchemistry rotated to matrix of genetic distance (left), and matrix based on Euclidean distance of edge microchemistry rotated to matrix of genetic distance (right). Arrows indicate the direction and magnitude of the rotation in space necessary to form a perfect match between both configurations.

In the final discriminant function analysis, only those individuals that were typed with microsatellite markers and otolith markers were used. Nine variables were entered in the model based on core microchemistry of this reduced dataset: Ba, Cd, Mn, Mg, Na, Co, Rb, Cu and Sr. The model showed an overall cross-validated classification success of 55 % with a maximal classification of 72 % observed for the sample from Kattegat and a minimal classification of 25 % for the Irish Sea sample (Fig. 6a). Although the assignment success was low for the Irish Sea, 36 % of the misclassifications were done to the neighbouring Celtic Sea. The genetic data were moderately successful in assigning samples from the Irish Sea (61 %), Celtic Sea (53 %) and Skagerrak (58 %) but for all other samples, genotypic assignment power was very low (< 35 %). The integration of data from both types of markers did not really improve the assignment power for the latter samples.

The linear discriminant function analysis based on the edge microchemistry data also retained nine elements: Ba, Mn, Mg, Na, Cd, Co, Sr, Rb and Zn and had an overall assignment success of 56 %. In general, otolith edge microchemistry showed more power for the traceability of fish to their capture location (52- 69 %) than genetic markers (19 - 58 %) (Fig. 6b). Only for the Irish Sea, genetic assignment was higher than otolith microchemistry (61 % and 25 %, respectively), although 32 % of the misclassifications based on edge microchemistry were allocated to the neighbouring Celtic Sea. Combining genotypes and otolith edge microchemistry improved the traceability power to the sampling location for the Kattegat, Belgian Coast and Celtic Sea to a level of 81%, 85 % and 69 % respectively (Fig. 6b).

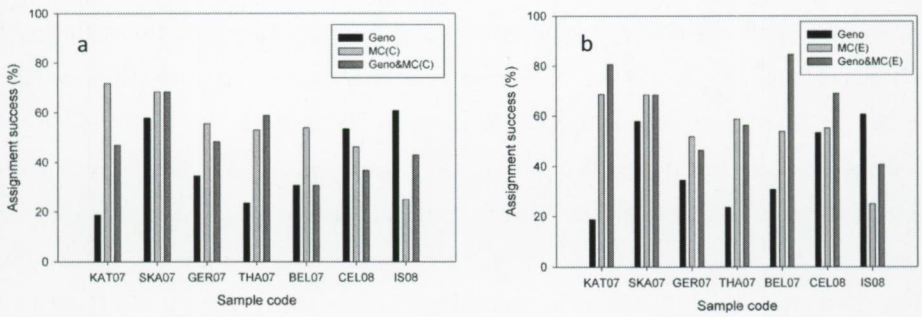

Fig. 6 Assignment success (%) calculated from the reduced dataset consisting of *Solea solea* individuals typed with both genetic and microchemistry markers. Results are shown for (a) analyses based on microsatellite data (Geno), microchemistry of core (MC(C)) and combined genotype-microchemistry core data (Geno&MC) and (b) analyses based on microsatellite data (Geno), microchemistry of edge (MC(E)), and combined genotype-microchemistry edge data (Geno&MC). For sample codes, see Table 1.

DISCUSSION

In this study we investigated whether the integration of genetic markers with ecologically relevant markers such as otolith microchemistry would improve our knowledge on the population structure and the traceability power of *Solea solea* in the North-East Atlantic Ocean. The assignment success to the fishing location was compared between both types of markers. At a large spatial scale (> 300 km), microsatellite data could discriminate sole from different regions, but at a smaller scale (< 150 km), the power of the genetic markers to reveal origin of fish was limited and the ecological markers provided more discrimination power.

Traceability

The results of this study showed that the traceability power to the three large geographic regions of the 'Baltic' (Kattegat/Skagerrak), the Southern Bight of the North Sea (UK, German Bight and Belgian coast) and Irish/Celtic Sea is generally higher with otolith edge microchemistry than with microsatellite markers. Genetic markers were however better at assigning fish to the Irish Sea, where otolith markers failed. Also at a smaller spatial scale, within the Southern Bight of the North Sea, otolith edge microchemistry performed better (59 % - 72 %) than genetic markers for classifying fish to their sampling location. The combination of information from genetic markers and otolith (edge) microchemistry improved the assignment power for the Kattegat, the Belgian Coast and the Celtic Sea.

Large differences in performance between both genetic assignment tests were observed, with higher success rates with the maximum likelihood test from Banks & Eichert (2000) implemented in WHICHRUN compared to the exclusion test from Rannala & Mountain (1997) in GENECLASS. Only 10 - 28 % of the samples were correctly assigned using the latter method, vs. 23 - 57 % using the first method. Differences in performance of genetic assignment methods have been found in other studies as well. Maudet et al. (2002) also observed much lower assignment accuracy with the Bayesian method applied in GENECLASS compared to the method used in WHICHRUN, especially in weakly differentiated populations (F_{ST} = 0.03). The same study also included the Bayesian method of Pritchard (2000), applied in the software STRUCTURE and identified this method as the most promising assignment method. In Chapter 1, we applied the Pritchard method to investigate population structure in sole, but we were unable to discriminate between different samples from the Southern Bight of the North Sea,

nor between Irish Sea and North Sea. Therefore this method was not included here. Also a study by Koljonen et al. (2005) found inconsistent results among assignment methods. The power of genetic assignment tests depends on the genetic differentiation among populations, the number of samples and sample size, the degree of polymorphism at the loci and the number of loci (Hansen et al. 2001; Hauser et al. 2006). In our study, either the number of loci or the level of genetic differentiation is too low for an accurate assignment. Typically, levels of differentiation required for a good assignment success are F_{ST} = 0.05 - 0.1 (Hauser et al. 2006), while F_{ST} values in our study were an order of magnitude lower (Chapter 1).

The success of otolith edge microchemistry as a traceability tool suggests that environmental differences exist and that adult sole remain long enough at a specific site to accumulate a site-specific element fingerprint. Before being able to use microchemistry data or genetic markers as a traceability tool, the temporal stability of the signal should be investigated.

Population structure

There was a positive correlation between the core microchemistry data and the genetic fingerprints, suggesting that both markers yield similar results concerning population structure in sole. However, the information that they contain is not completely redundant. An important issue for fisheries management is the extent to which geographically separated 'populations' are connected over evolutionary and ecological timescales. While genetic markers provide an estimate of dispersal across multiple generations (evolutionary time scale), otolith data provide information across an individuals' lifetime and reflect variation at ecological time scales (Campana 1999). Genetic markers are also influenced by the population history, effective population size, besides spatial variation. The absence of significant genetic differences could therefore also result from the recent re-colonization history and the high effective population size of sole (see Chapter 7). Spatial differences in otolith chemistry result from differences in water chemistry and physiology.

The high classification success based on otolith core microchemistry, mainly for samples from the Baltic region, suggests limited levels of exchange between the Kattegat and the North Sea. This observation is consistent with a population genetic study on *Solea solea* at the same spatial scale, where spatial genetic differentiation was observed between the Baltic region (the Kattegat and the Skagerrak) and the North Sea (Chapter 1). Within the

North Sea (ICES area IV), there is no strong evidence for the existence of multiple discrete populations. Genetically, all samples were very similar, resulting in a low assignment power based on the ten microsatellite markers used in our study. Also based on their otolith core microchemistry composition, assignment success within the North Sea was never higher than 60 % and assignments were frequently done to one of the other North Sea samples, suggesting either some degree of connectivity or a lack of discrimination power. Even though the assignment success for the samples from the Irish Sea was low using otolith core microchemistry, misclassifications were mainly done to the Celtic Sea. Moreover, this population had a high assignment success with genetic markers. Even if genetic differentiation (F_{ST}) is not always significant (Chapter 1), our results suggest a lower level of genetic exchange between Irish Sea and North Sea, corresponding to the current management units. This is consistent with tagging studies of sole in the Irish Sea and Bristol Channel, showing mainly local recruitment and limited movement of sole outside the management units (Horwood et al. 1993). Although an acceptable number of microsatellite markers was used in our study, the use of SNP markers will possibly improve the resolution power of genetic differentiation (Waples & Gaggiotti 2006).

To verify the existence of distinct spawning units of sole in the North-East Atlantic Ocean, temporal replicates should be analyzed for all locations, in order to exclude the possibility of temporal variation confounding the spatial patterns (Gillanders 2002b; Hauser & Carvalho 2008).

Conclusion

Our results show that otolith microchemistry provided similar results as microsatellite genotypes at a large spatial scale (among basins), suggesting that environmental differences were strong enough to build up a distinctive fingerprint and populations were sufficiently isolated to differ genetically. At a smaller spatial scale, high gene flow or the high effective population size is likely preventing strong genetic differentiation, while otolith chemistry could still detect spatial differences among groups, which could be relevant to fisheries management. These results highlight the power gained by a multi-marker approach depending on the required resolution scale, enabling the use of even subtle variations between populations for traceability purposes.

ACKNOWLEDGEMENTS

Our research was supported by the European Community's Seventh Framework Program under contract no. KBBE-213399 (FishPopTrace) and by the WESTBANKS project, which is supported by the Belgian Science Policy (BELSPO; contract no. SD/BN/01A). The authors would like to thank J. Guelinckx, E. Diopere, S. Verherstraeten, K. Vancampenhout (KULEUVEN), U. Damm and N. Rohlf (ISH-BFAFI), E. Nielsen and colleagues (DTU-AQUA), ILVO-Fisheries and the crew of RV Belgica and RV Zeeleeuw, for their help with the collection of the samples. Thanks to J. Skadal (UIB), O. Tumyr and J. Kosler (Centre of Geobiology, UIB) for their help with the operation of the laser and ICPMS. Acknowledgements to BeNCoRe network and Research Foundation Flanders (FWO Vlaanderen) for a travel grant. E.C. acknowledges a grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). G.E.M. and J.A.M.R. are post-doctoral researchers funded by the Fund for Scientific Research (FWO Vlaanderen).

SUPPLEMENTARY MATERIALS

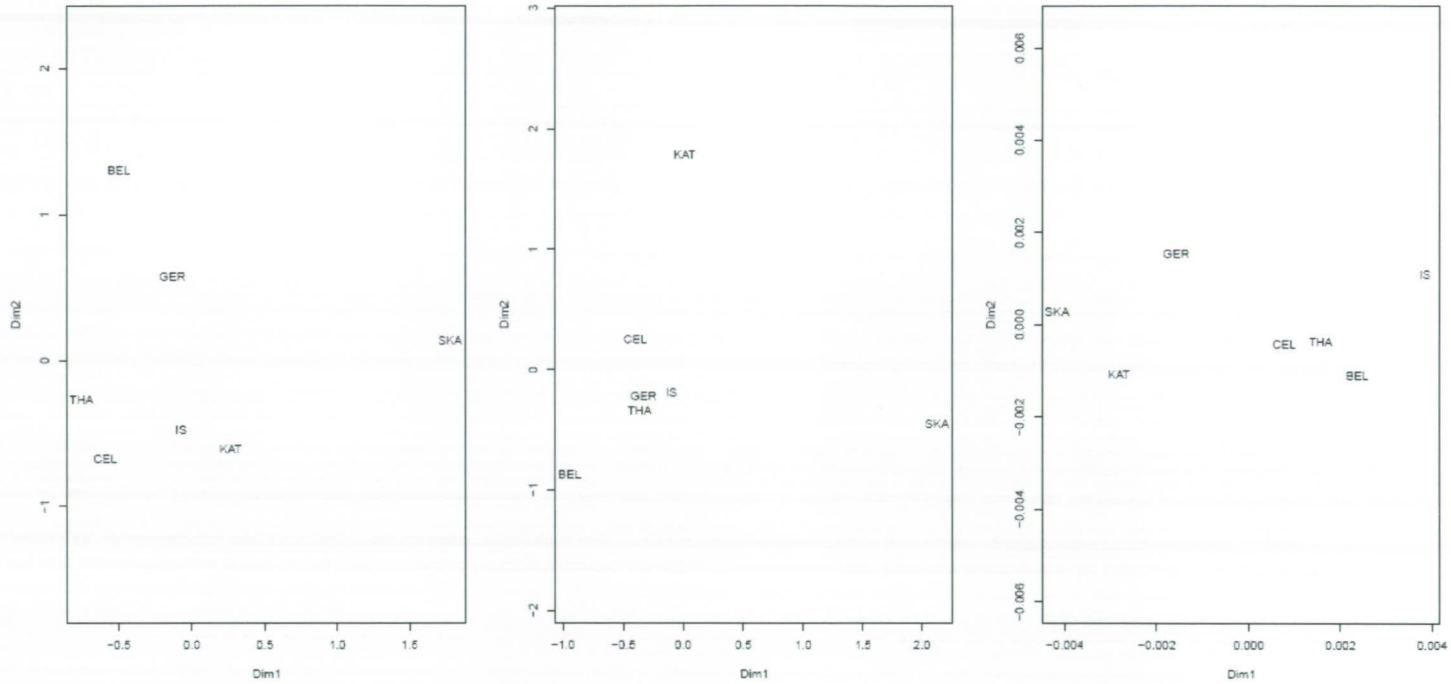

Fig. S1 Two-dimensional presentation of a classical multidimensional scaling analysis of the seven samples based on Euclidean distance of core microchemistry (left), Euclidean distance of edge microchemistry (centre) and pairwise F_{ST} (right). Letters present sample codes (see Table 1).

Genetic stability in *Solea solea*

Part II

Chapter 6

Influence of DNA isolation from historical otoliths on nuclear-mitochondrial marker amplification and age determination in an over-exploited fish, the common sole *(Solea solea* L.)

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ABSTRACT

Historical otolith collections are crucial to assess the evolutionary consequences of natural and anthropogenic changes on the demography and connectivity of commercially important fish species. Hence, it is important to define optimal protocols to purify DNA from such valuable information sources, while avoiding any damage to the physical structure of the otolith. Before being able to conclude on the harmlessness of a method, it is important to validate protocols on different kinds of otoliths, by testing purification methodologies under standardized conditions. Here we compare the effect of two DNA extraction methods on the success in identifying the age in an overexploited marine fish, the common sole *(Solea solea* L.). To ensure optimal future population genetic and demographic analyses, we assessed DNA quantity and tested the DNA quality by investigating the amplification success of a mitochondrial and nuclear marker. Our results show that the choice of the DNA extraction method had a significant effect on the success of using these otoliths in age and growth analyses. Standard commercial and published protocols resulted in a severe damaging of the otolith structure, hampering accurate preparation and analyses of the morphological structures of the otoliths. Shortening the lysis time and lowering the EDTA and SDS concentration turned out to be beneficial for the stability of otolith structure, while maintaining an overall high DNA quality measured through PCR amplification success. We therefore recommend that care should be taken when choosing the extraction method for a molecular study on archived samples, in order to enable the maximal use of information embedded in historical material.

Published in Molecular Ecology Resources (2009), 9, 725-732

INTRODUCTION

Many exploited fish stocks around the world show signs of genetic or phenotypic changes in life-history traits caused by natural (e.g. climate change) and anthropogenic (e.g. overfishing) factors (Kuparinen & Merila 2007; Law 2007). These changes can have a profound impact on the demography and connectivity of commercially important fish species. Hence, recent recommendations from the European Union (ICES 2005b) urge for the assessment of fisheries and climate induced changes in declining marine stocks. While the problem of climate change requires a global management strategy in the long-term, indications of overfishing require a rapid management response to reverse the stock decline and possible irreversible evolutionary changes. Recent studies using historical fisheries data have indeed strongly suggested fisheries-induced evolution of important life-history traits, such as age and size at maturation, growth or reproductive effort in exploited fish populations (Barot et al. 2004; de Roos et al. 2006; Morita & Fukuwaka 2007). Most evidence has been gained from the analysis of phenotypic data in numerous marine fishes, indicating significant changes in the maturation reaction norms, i.e. an individual's probability to mature at a given size and age (Dieckmann & Heino 2007; Grift et al. 2003; Mollet et al. 2007).

The evolutionary consequences of fisheries-induced evolution can also be studied at the DNA level or even in terms of quantitative genetic parameters. Besides a reduction in the census population size, (over)exploitation will most likely lead to a reduction in the genetic effective population size (N_e), the number of individuals that successfully propagate their genes to the next generation (Hauser et al. 2002; Hoarau et al. 2005; Turner et al. 2002). Additionally, adaptive responses can occur in exploited marine organisms such as a decrease or shift in genetic variability at important functional genes related to maturity and growth (Wenne et al. 2007). Because these heritable genetic changes might be irreversible and adverse for a species it is useful to partition total phenotypic variance in a plasticity and a genetic component (Kuparinen & Merila 2007). A suitable strategy is the joint analysis of phenotypic and genetic data from contemporary populations, compared with a reference situation (preferably before the population decrease). For this purpose, ideally the same historical otoliths used for phenotypic analyses should be used as a valuable source of DNA, since dried tissue on the otolith surface still contains biological material for genetic analyses (Hutchinson et al. 2003).

Unfortunately, the DNA from archived material is usually degraded and only present in very small amounts. Even though sagittal otoliths exist in pairs, often one of both otoliths has been used in a destructive way for ageing the fish, e.g. break and burn method (Christensen 1964), leaving only one otolith for all other research purposes (such as microchemistry, DNA analyses and growth analyses). Therefore the need exists to develop reliable and simple methods to collect a maximum of information from only one otolith without damaging it. Currently, several methods exist for the successful extraction of DNA from old scales or otoliths (Table 1). In few studies though, the otolith was subsequently used for other purposes than molecular work. When choosing for an appropriate DNA extraction method, one should take into account the yield of purified DNA, the cost of extraction and the quality of purified DNA. In the case where there is only enough tissue for a single extraction, extraction protocols which give a high yield of high quality DNA extract are to be preferred (Paabo et al. 2004; Poulsen et al. 2006). However, if a single otolith needs to be used for DNA extraction and subsequent ageing, one should be cautious not to destroy the otolith's structure. Hence an optimal trade-off exists between DNA quality and otolith preservation. In a study of Hutchinson et al. (2003) a nuclear amplification success of 80-100 % was reached for historical cod otoliths using the extraction protocol of Hutchinson et al. (1999). The same protocol was successfully followed by Ruzzante et al. (2001) for the DNA extraction from cod otoliths and later on by Hoarau et al. (2005) on historical plaice otoliths. Other authors have successfully used the extraction method by Estoup et al. (1996) to study population stability (Jakobsdottir et al. 2006; Poulsen et al. 2006). Also various commercial DNA extraction kits are available but they are basically very similar in use. They are less time consuming than the methods mentioned above, but the main disadvantages with these are the higher cost price (especially for large sample sizes) and the patented secret composition of the reagents. The Nucleospin tissue kit (Nucleospin, Machery Nagel) was selected in this study since previous work on the study species *Solea solea* showed that a good DNA quality could be obtained for contemporary samples (Cuveliers EL, unpublished data).

Table 1 Overview of published literature using DNA extraction protocols in archival fish material.

Author	Species	Material	Protocol	Purposes
Miller & Kapuscinski 1997	*Esox lucius*	scales	Authors protocol	Molecular work
Nielsen *et al.* 1997	*Salmo salar*	scales	Phenol/chloroform	Molecular work
Nielsen *et al.* 1999	*Salmo salar*	scales	Comparison of several methods	Molecular work
Tessier & Bernatchez 1999	*Salmo salar*	scales	Estoup *et al.*1996	Molecular work
Hutchinson *et al.* 1999	*Gadus morhua*	otoliths	Authors protocol	Molecular work
Adcock *et al.* 2000	*Pagrus auratus*	scales	Salting-out method of Bruford *et al.* 1992	Molecular work
Ruzzante *et al.* 2001	*Gadus morhua*	otoliths	Hutchinson *et al.* 1999	Molecular work
Hauser *et al.* 2002	*Pagrus auratus*	scales	Salting-out method of Bruford *et al.* 1992	Molecular work
Koskinen *et al.* 2002	*Thymallus thymallus*	scales	Commercial extraction kit (Qiagen)	Molecular work
Saïsa *et al.* 2003	*Salmo salar*	scales	Commercial extraction kit (Qiagen)	Molecular work
Hoarau *et al.* 2005	*Pleuronectes platessa*	otoliths	Hutchinson *et al.* 1999	Molecular work
Jakobsdottir *et al.* 2006	*Gadus morhua*	otoliths	Estoup *et al.* 1996	Molecular work
Lucentini *et al.* 2006	*Esox lucius,*	scales	Comparison of several methods	Molecular work
Hansen *et al.* 2006	*Salmo trutta*	scales	Nielsen *et al.* 1999	Molecular work
Poulsen *et al.* 2006	*Gadus morhua*	otoliths	Estoup *et al.* 1996	Molecular work
Heath *et al.* 2007	*Plectropomus leopardus*	otoliths	Wizard Genomic Purification Kit	Molecular work & ageing

In a recent study, Heath et al. (2007) tested the effects of DNA extraction on the ageing success in the otoliths of coral trout (*Plectropomus leopardus)*. They did not find any significant difference between untreated and treated otoliths and concluded that commercial DNA extraction kits were safe. However, the composition of commercial kits can differ, especially in the chemical components such as Ethylene Diamine Tetraacetic Acid (EDTA) and Sodium Dodecylsulphate (SDS). Additionally, otoliths from different species are likely to differ in their propensity to damage during the extraction protocol, due to differences in shape, composition and size.

In the present study, we wanted to compare the effect of different historical DNA extraction methods on (1) the age reading success of otoliths, (2) the DNA quantity after purification and (3) the amplification success (DNA quality) measured as nuclear and mitochondrial PCR efficiency in the common sole (*Solea solea* L.). The common sole is a commercially valuable species suffering from a strong decrease in population size in the last decades (Hutchings 2000; Hutchings & Baum 2005, ICES 2007) and showing obvious shifts in life history traits (Mollet et al. 2007). We finally provide some general recommendations to jointly analyse phenotypic and molecular data from the same archived material.

MATERIALS AND METHODS

Otolith samples

To assess the effect of the DNA extraction protocol on fish age determination, batches of five to nine otoliths were selected from common sole otoliths collected in 1965 and 1978. Due to limited resources of the archived material available for testing, not all otoliths were from the same year. The otoliths were part of a large collection (1957-2001) belonging to the Institute for Marine Resources and Ecosystem Studies of The Netherlands (Wageningen IMARES). They were all collected from commercial landings of sole in the North Sea and all samples have been stored dry in paper bags.

DNA extraction protocols and quantification

DNA was extracted from the otolith's adhering tissue, using two different DNA extraction methods, a specific protocol for otolith DNA extraction and a commercial kit (Table 2). In method A, otoliths were treated using the extraction protocol described by Hutchinson et al. (1999). We initially tested the published method (5 h incubation, 2 % SDS and 10 mM EDTA). The reagents SDS and EDTA are included to break up the lipids in the membranes and to reduce the effects of the DNAses during digestion, respectively (Sambrook & Russel 2001). After an initial testing procedure, the protocol was slightly modified by (a) decreasing the concentration of SDS from 2 % to 0.5 %, (b) decreasing the incubation time from 5 h to 3 h, 2 h and 1 h and (c) decreasing the concentration of EDTA from 10 mM to 1 mM. In method B, DNA was extracted using the commercial DNA extraction kit 'Nucleospin Tissue kit' (Nucleospin, Machery Nagel) according to the manufacturer's instructions. The otoliths were incubated for 1 h, 3 h or overnight. DNA was resuspended in 100 µl ultrapure water in all the methods except for the initial method of Hutchinson et al. (1999) (Method A1), where the final elution step was performed with 50 µl ultrapure water, as described in the original article. After removing the otoliths from the lysis suspension, they were rinsed with distilled water and placed back in the respective envelope for later embedding and age reading. DNA quantity was determined with a fluorescent dye binding assay using PicoGreen® dsDNA (Invitrogen) as a quantification reagent and 10 µl of otolith DNA extract. The measurements were performed according to the manufacturer's instructions with an Infinite M200 microplate reader with a detection limit of 20 pg dsDNA in a 200 µl assay volume (TECAN

Trading AG). Statistical differences in DNA quantity between the methods were evaluated with a Kruskal-Wallis ANOVA test (significance level alpha = 0.05) (Statsoft, 2008).

Molecular analyses

Considering the much higher copy-number per cell of mitochondrial DNA compared to nuclear DNA copy numbers in archived samples (Paabo et al. 2004), we tested the quality of PCR templates (DNA integrity) for both classes of molecular markers. DNA quality was expressed as nuclear and mitochondrial PCR amplification success. Nuclear DNA amplification was performed using primers for a single microsatellite DNA locus F8-IIGT15 (AF173852) with a fragment size range of 132-158 bp, known to give good amplification in contemporary samples (Iyengar et al. 2000). The forward primer (5'-ATCATACCAAGTGTGAGACC-3') was labelled with the VIC-dye, while the reverse primer (5'-GCTGATTTACTGTACTTGGC-3') remained unlabeled. Mitochondrial DNA amplification was performed using the cytochrome *b* primers CB1bis-F (5'-TACGTCCTCCCCTGAGGACAGATATC-3') and SosoCytB3-R (5'-CCTGTTTCGTGAAGGAAAAATAA-3'), yielding a 223 bp long PCR fragment (based on a 386 bp fragment from Volckaert et al. (unpublished data) (GENBANK accession nos. AY970706-AY970781). PCR amplification consisted of an initial denaturation at 95°C, annealing at 45°C (mtDNA primers) or 61°C (Microsatellite primers), elongation at 72°C, 40 cycles. To avoid contamination, negative controls were used at every step from extraction to genotyping. The quality of the PCR products was tested by running the amplicons first on a 1% agarose gel and then on an automatic capillary sequencer ABI 3130 AVANT (Applied Biosystems), while recording the relative peak intensity of the microsatellite locus and the sequence nucleotide quality values of the cyt *b* fragment.

After DNA extraction the otoliths were sent back to IMARES for further treatment (embedding, sectioning and age reading) required for later age determinations and growth back-calculations.

Age reading quality

Transverse sectioning is generally considered to be the best preparation method for the otoliths of many species, including sole. The method was first described by Bedford (1983) and has been adopted and refined in many laboratories (e.g. van Beek et al. 1997). Crucial for the precision of ageing and back-calculation of growth rates when using this method is that the section is taken precisely through the nucleus. Before being embedded in resin, the otoliths are lined up with their nucleus exactly on the cutting line. This is enabled by the fact that the opaque nucleus is visible in the largely transparent otolith. However, any treatment altering the transparency of the otolith (such as DNA extraction procedures) can severely hamper the detection of the nucleus. Taking a section precisely through the centre of the otolith does indeed not ensure that the section is taken through the nucleus, neither in the asymmetrical otolith (which is obvious), nor in the symmetrical otolith (which is less obvious). Due to drastic alterations in otolith quality at the start of the project, we decided to examine the effect of different DNA extraction conditions based on two protocols, described above and listed in Table 2, on the transparency of sole otoliths. Transparency of the otoliths and hence the possibility of using these otoliths for age determinations and growth back-calculations, was rated by two independent readers as good, moderate or bad. "Good" otoliths appear to be unaffected by the DNA extraction procedure, "moderate" otoliths show clear signs of deterioration but the nucleus is still (partly) visible, and "bad" otoliths are severely corroded making it impossible to accurately section the otolith precisely through the nucleus (Fig. 1). The transverse sections can be examined for tell tale signs of a missed centre of the nucleus, but it remains impossible to detect if the nucleus is missed entirely. Therefore all "bad" otoliths should be rejected from further age determinations or growth back-calculations. "Moderate" otoliths may only be considered for these analyses if the sections show no signs of inaccuracy combined with a high degree of certainty on the detection of the nucleus prior to sectioning.

RESULTS

Molecular analysis

Method of Hutchinson et al. *(1999) (methods A1-A5)*

The DNA extracted using the original protocol of Hutchinson et al. (1999) (Method A1) showed an amplification success rate of only 33 % for the nuclear marker and 60 % for the mitochondrial marker (Table 2). When decreasing the concentration of SDS to 0.5 % (methods A2 to A5), amplification success of the nuclear marker increased up to 100 %. The cytochrome *b* fragment still amplified only in two out of five samples when only SDS concentration was lowered. Decreasing the lysis time to less than 5 h had only a positive effect on the amplification success for the mitochondrial marker, although further decreasing the lysis time to 3 h, 2 h or 1 h did not seem to further improve the DNA quality (Table 2).

Nucleospin methods (B1-B3)

The commercial method yielded an amplification success of 89 % for the microsatellite marker and 56 % for the mitochondrial marker, if the lysis was done overnight (method B3). The amplification success of both nuclear and mitochondrial marker increased up to 100 % with a lysis time of only 1 hour.

General quality and quantity

DNA quantity ranged from 0.09 to 13 $ng.\mu l^{-1}$ and was significantly different between extraction methods (Kruskal-Wallis ANOVA, $p < 0.05$). The mean DNA quantity was highest in method A2 and lowest in method B3 (Table 2). When run on an automatic sequencer, the microsatellite amplification peak density was generally high and comparable with contemporary samples (peak intensity > 2000) and sequence quality was high (mean quality value per sequence of 40).

Age reading quality

Visual inspection rapidly showed that the two standard DNA extractions (method A1 and B3) appeared to corrode the surface of the otolith, thus affecting its transparency. Different DNA extraction methods varied in the degree of deterioration of the otolith. The left panels in Fig. 1 show the highly corrosive effect of the original Hutchinson et al. (1999) method with a long incubation time (5 h) and a high concentration of EDTA (10 mM). Damage to the otoliths does not decrease if only the concentration of SDS is reduced (from 2 % to 0.5 %), but major improvement is achieved with a combined decrease of the incubation time (1-3 hours) and the EDTA concentration (1 mM) (Table 2, right panels in Fig. 1). Decreasing the incubation time to less than 3 h did neither seem to further improve the transparency of the otoliths or the amplification success rates. The commercial Nucleospin method (method A3) seemed also highly corrosive if a long incubation time (overnight) is applied. Otoliths treated with this method (B3) looked similar to those on the left panel of Fig. 1. Although the damage is reduced with a shorter incubation time (1-3 hours), this method was clearly more destructive than the Hutchinson method at low EDTA concentrations (Table 2).

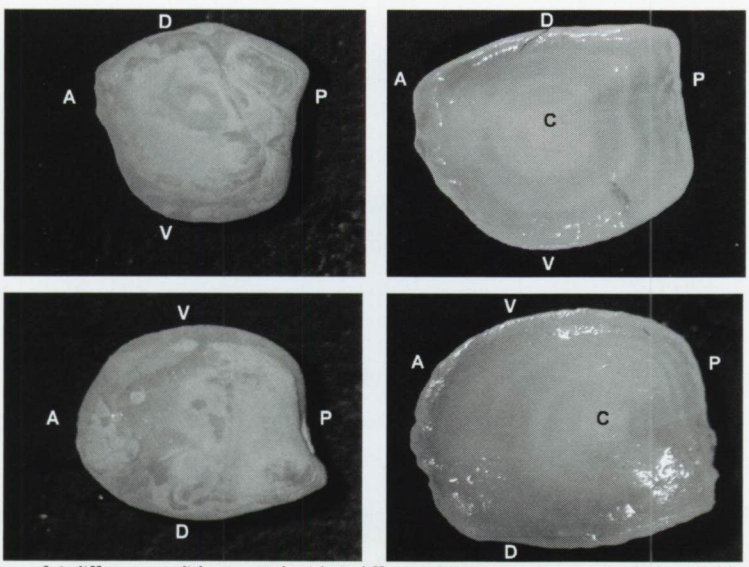

Fig. 1 Images of 4 different otoliths treated with 2 different DNA extraction methods (see Table 2). The left otoliths were treated with method A2 and clearly show signs of corrosion. The right otoliths were treated with an adjusted protocol (method A3) and appear to be undamaged. The top otoliths are asymmetrical otoliths (top right panel clearly shows that the nucleus is not in the centre of the otolith) and the bottom otoliths are symmetrical otoliths (bottom right panel shows that the nucleus is close to but not entirely in the centre of the otolith). (A= anterior side; P= posterior side; V= ventral side; D= dorsal side; C= core area)

Table 2 Extraction method, incubation time, concentration of EDTA, concentration of SDS, microsatellite success rate (MSS) and cytochrome *b* success rate (CBS) with number of samples between parenthesis, DNA concentration (mean and standard deviation), Otolith quality (B=bad, M=moderate, G=good).

Method	Author	Incubation time	EDTA (mM)	SDS (%)	MSS (%)	CBS (%)	DNA Conc ($ng.\mu l^{-1}$)	Otolith quality (transparency)
A1	Hutchinson	5 h	10	2	33 (9)	60 (5)	0.998 ± 1.191	B (8B+1M)
A2	Hutchinson	5 h	10	0.5	100 (9)	40 (5)	3.203 ± 4.899	B (9B)
A3	Hutchinson	3 h	1	0.5	80 (5)	100 (5)	2.415 ± 1.304	G (4G+1M)
A4	Hutchinson	2 h	1	0.5	80 (5)	100 (5)	2.502 ± 2.875	G (3G+2M)
A5	Hutchinson	1 h	1	0.5	80 (5)	100 (5)	1.436 ± 0.916	G (3G+1M)
B1	Nucleospin	1 h	unknown	unknown	100 (5)	100 (5)	0.182 ± 0.086	M (2B+2M+1G)
B2	Nucleospin	3 h	unknown	unknown	80 (5)	100 (5)	1.379 ± 0.824	M (2B+2M+1G)
B3	Nucleospin	Overnight	unknown	unknown	89 (9)	56 (9)	0.789 ± 0.774	B (9B)

Discussion

Our results on sole clearly showed differences in corrosion and subsequent ageing ability for otoliths treated with different extraction methods. A modified published method of Hutchinson et al. (1999), namely by reducing the EDTA concentration, SDS concentration and incubation time, caused little or no damage to the otoliths and yielded good amplification rates. The original Hutchinson et al. (1999) method and the commercial Nucleospin method caused severe damage to the otoliths, resulting in the loss of almost all of them for further analyses of age and growth rates. For all tested methods and incubation times, except the initial Hutchinson et al. (1999) method, the DNA purity remained sufficiently high for nuclear PCR success. The lower PCR success observed using the original method could be due to the smaller elution volume, since eluting the DNA extract can have a positive effect on PCR success (Sambrook & Russel 2001). In the treatments with a lower concentration of EDTA, also a good amplification of the mitochondrial marker could be obtained. The peak quality and sequence nucleotide quality values remained constant until 1 h incubation time, indicating that the low amount of DNA covering the dried otoliths is quickly transferred to the lysis suspension. This is important to ensure DNA of sufficient quality and to avoid subsequent artefacts during PCR amplifications. Other studies on historical otoliths of flatfish for instance did show some evidence of Hardy Weinberg disequilibrium (Hoarau et al. 2005).

This might be explained by the incidence of null alleles and requires additional control steps before being able to use such genetic information (Wandeler et al. 2007). Additionally, our results confirm that sufficient DNA is available for as well nuclear as mitochondrial analyses, an important issue as multi-marker analyses increase the confidence of estimates of population size decrease and human-induced evolution. Although the measured DNA concentrations were very low, amplification success for both genetic markers were high and relative differences in DNA quantity could be observed between treatments.

Given that various biological, physical and chemical factors affect the DNA quality, the degradation of DNA from archived otoliths increases with time (Wandeler et al. 2007). Although it would have been optimal to perform all the tests on otoliths from the same year, our data did not suggest a higher amplification success in the few samples of 1978 compared to 1965. Moreover, all samples were stored under the same conditions so there are no indications that the difference in storage time (13 years) would have any influence on the otolith structure.

The damaging of otoliths is however in strong contrast with a recently published study on coral trout, where no influence was observed on the quality of otolith age reading after a standard commercial DNA purification treatment (Heath et al. 2007). They concluded that the study of archived material can safely be preceded by a DNA extraction step using a commercial purification kit. The destructive effect of the DNA extraction on the otolith structure in some of the methods is most likely due to the decalcifying nature of certain compounds in the extraction solution. Indeed, most digestion buffers contain some EDTA to bind metal ions and to reduce the effects of the DNases. In other research disciplines, EDTA is also used at high concentrations (e.g. 0.5 M) for etching to improve reading ability with Scanning Electron Microscopy (Campana & Neilson 1985). Due to the decalcifying characteristics of this compound (because of its chelating power towards calcium, especially if the pH of the solution is above pH = 6), a destructive effect could be expected with otoliths, if the concentration of EDTA is too high or incubation times are too long (Shiao et al. 1999). Our study clearly proves that this expectation is correct; reduction of the EDTA concentration and the incubation time strongly reduced the corrosive effect of DNA extraction.

While age determination and back-calculation of growth rates were virtually impossible when using the original Hutchinson et al. (1999) method, little to no damage occurred when using the modified Hutchinson et al. (1999) method. Most DNA preparations also contain SDS to break up the lipids in the membranes, so to free the DNA from the cell. There was no reason to assume that this detergent, used for the denaturation of proteins, has destructive effects on otolith structure and this was confirmed in the present study.

Although various studies used otoliths for DNA extraction and reconstruction of historical events (Table 1), the combined use of the same otolith for molecular and phenotypic analyses is novel (Heath et al. 2007). As such it is important to inform the scientific community when problems may arise with standard methods, possibly damaging unique collections. No method is safe until thoroughly tested on the target species, as the characteristics of the otoliths (shape, thickness, $CaCO_3$ composition, opacity and transparency) are species-specific and should be considered (Campana & Casselman 1993). The larger the otolith and especially its nucleus (for instance in cod, E. Nielsen Pers. Comm.), the lower the expected effect and the more reliable the nuclear section. However, in small and transparent otoliths, such as in flatfishes, destruction can happen very quickly and irreversibly.

To conclude, we recommend that care should be taken when choosing an appropriate DNA extraction protocol in order not to damage the otolith structure for complementary analyses. This warning is valid for all studies using archived samples that require their availability for complementary treatments. The extraction of high quality DNA without damaging the otolith structure will open many new possibilities for multidisciplinary analyses and a better understanding of evolutionary responses of fish stocks under natural and human-induced changes.

ACKNOWLEDGEMENTS

Our research was supported by the European FP6-STREP project FinE (Fisheries Induced Evolution, nr: SSP-2006-044276). We thank P. Groot, I. Pennock and S. Tribuhl from Wageningen-IMARES for the otolith images and the assessment of the otolith quality. We thank B. Hutchinson for his useful comments during optimization of the protocol. E.C. acknowledges a grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). G.E.M. is a post-doctoral researcher funded by the Fund for Scientific Research (FWO Vlaanderen).

Counting fish...

Chapter 7

Temporal genetic stability and high effective population size in the North Sea sole over a 50-year time period coinciding with high fishing pressure

Cuveliers E.L., Volckaert F.A.M., Rijnsdorp A.D., Larmuseau M.H.D. & Maes G.E.

ABSTRACT

Heavy fishing and other anthropogenic influences can have profound impact on a species' resilience to harvesting. Besides the decrease of the census and effective population size, strong declines in mature adults and recruiting individuals may lead to almost irreversible genetic changes in life-history traits. Here, we investigated the evolution of genetic diversity and effective population size in the heavily exploited sole (*Solea solea*), through the analysis of historical DNA from a collection of 1379 sole (*Solea solea*) otoliths dating back from 1957. Genetic diversity inferred from 11 microsatellite markers showed a remarkable stability over a period of 50 years of heavy fishing. Point and temporal estimates of effective population size (N_e) were always higher than 1000, suggesting that genetic drift is probably not strong enough to significantly decrease the neutral diversity of this species in the North Sea. However the ratio of effective population size to the census size (N_e/N_c) was very small (10^{-5}), suggesting that only few adults contribute to the next generation. The low N_e/N_c ratio is most likely caused by the large variance in reproductive success. Because strong evolutionary changes in age and size at first maturation have been observed for sole, changes in adaptive genetic variation should be further monitored to detect human-induced selection.

INTRODUCTION

Heavy fishing and other anthropogenic influences can strongly impact a species' resilience to harvesting (Hauser et al. 2002; Kenchington et al. 2003). The direct effects of fishing are obvious; it causes an increase in mortality of both target and non-target species. Fishing may also shift the size and age structure or the sex-ratio of a stock by selectively removing part of the population, which on its turn can have profound effects on the reproductive output (Jennings et al. 2001; Kenchington et al. 2003). Although life history traits are plastic and change in response to the environment, there is growing evidence that exploitation might also cause irreversible evolutionary changes in fish populations. Many of the life history traits are heritable and will therefore evolve in response to fishing pressure (Heino 1998; Law 2000). For several marine fish, a trend has been observed towards earlier sexual maturation at smaller size (Conover et al. 2005; Jørgensen et al. 2007; Kuparinen & Merilä 2007; Mollet et al. 2007). Since fishing reduces population size it may also reduce genetic variation if there are not enough individuals to maintain the full range of variability (Hauser et al. 2002; Lage & Kornfield 2006). This will primarily result in the loss of alleles due to genetic drift or in further instance even in the loss of heterozygosity (Hoelzel et al. 2006; Allendorf et al. 2008). Low genetic variation has been associated with inbreeding depression, the accumulation of deleterious alleles and reduced adaptive potential (Frankham et al. 2002).

Generally, genetic diversity only starts to decline when population numbers are becoming very small (N_e < 500, (Franklin 1980)). Since overexploited fish stocks may still include millions of adult fish, it has been assumed that the loss of genetic diversity should remain minimal (Hutchings & Reynolds 2004). Nevertheless, several studies have indicated a reduced genetic diversity in overexploited stocks, suggesting that the number of fish that are reproducing and contributing to diversity is actually much smaller than the total numbers present (Hauser et al. 2002; Hutchinson et al. 2003). The maintenance of genetic diversity indeed depends on the effective population size (N_e), a challenging key parameter to measure for marine conservation genetics (Waples et al. 2008). Genetic methods for estimating N_e in natural populations are most often based on the measurement of variance in allele frequencies between generations ('variance effective size'). One of the most used and reliable methods when multiple samples are available, is the temporal method where

the harmonic mean of N_e during the sampling period is estimated based on the observed rate of genetic drift (Waples 1989; Wang 2005). This estimate gains more accuracy with an increasing number of generations between the temporal samples (Wang 2005; Waples & Yokota 2007).

Evolutionary effects of exploitation can be investigated by comparing genetic diversity in samples before and after periods of intensive exploitation (Hauser et al. 2002; Saisa et al. 2003). To assess with accuracy the recent historical loss of genetic diversity in fish stocks due to climatic, environmental and anthropogenic influences, one needs to compare the contemporary level of genetic variation with a historical baseline level (representative for a lower anthropogenic impact), hence avoiding the shifting-baselines trap (Pinnegar & Engelhard 2008). An analysis of continuous time-series of historical material rather than sporadic old samples may thus increase the confidence in genetic estimates of population sizes. Archived otoliths collected during a century of fisheries science form an ideal source of DNA to assess changes in genetic diversity over time (Nielsen & Hansen 2008). Although these earstones, originally used for age determination, are acellular, DNA can be extracted from the adhering dried tissue with great success (Hutchinson et al. 1999, Chapter 6).

From the 1960s onwards, large beam trawlers were operating in the North Sea leading to a concomitant increase in exploitation level (Millner & Whiting 1996; Rijnsdorp et al. 2008). Sole (*Solea solea* L.), together with plaice (*Pleuronectes platessa* L.) and cod (*Gadus morhua* L.), has always been a target species of the North Sea beam trawl fisheries. For the latter two species either a low effective population size or fluctuations in genetic diversity have been observed and related to overexploitation (Hutchinson et al. 2003; Hoarau et al. 2005). Additionally, indications of fisheries-induced changes in maturation reaction norms were observed in North Sea plaice and sole, namely a shift towards maturation at earlier age and smaller size (Grift et al. 2003; Rijnsdorp et al. 2005; Mollet et al. 2007; van Walraven et al. 2010). As sole represents one of the most important resources in the North Sea, with an average yearly landing of more than 16 000 tonnes in the last decade and fishing mortalities above the precautionary approach reference point ($F > Fpa = 0.4$), there is an urgent need to assess the evolutionary consequences of such harvesting pressure on the level of genetic diversity and thus the long-term evolutionary resilience of sole.

Here, we analyse an archived collection of sole otoliths (1957-1995) combined with contemporary samples (2007) to study temporal changes in neutral genetic variation in the North Sea and to estimate the effective population size with various methods. Thanks to the availability of accurate individual ages, it was possible to investigate the fluctuations in genetic variability between sampling years with a mixed-cohort design and between single year classes/age groups over a 50 year period. We aimed at investigating whether the increase in fishing pressure from the 1960s onwards is changing the genetic characteristics of the North Sea sole. We discuss alternative hypotheses on low N_e/N_c ratio in heavily exploited marine fish.

MATERIALS AND METHODS

Samples

Historical sole otoliths from the North Sea were obtained from a collection curated by IMARES (Institute for Marine Resources and Ecosystem Studies, The Netherlands). Otolith samples were collected during research surveys or commercial sampling in January-April covering the Southern North Sea in the years 1957 to 1995 and conserved dry in paper envelopes (approximate positions - Fig. 1). All selected otoliths were aged according to standard methodologies (Millner & Whiting 1996). To maximize the genetic information content and to minimize the sampling bias, we aimed at jointly clustering individuals in separate cohorts and age groups, besides sampling years. In total the genetic analysis was done on 1159 historical samples, including 215 aged samples from the 1950s ('NS50'; sampling years 1957-1959), 205 samples from the 1960s ('NS60'; sampling years 1966-1967), 205 samples from the 1970s ('NS70', sampling years 1971-1974), 404 samples from the 1980s (NS80, sampling years 1984-1985-1987) and 130 samples from 1995 (NS95) (For details on age composition of the samples, see Supplementary Materials Table S2). In addition, contemporary samples were collected in 2007 during research surveys off the Thames, on the Norfolk Banks and off the Belgian Coast (Fig. 1).

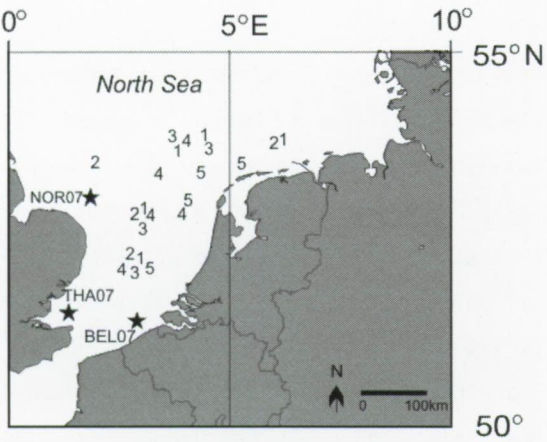

Fig. 1 Sampling locations (approximate positions) for *Solea solea*. Asterisks indicate contemporary samples; numbers indicate historical sampling locations: 1='NS50', 2= 'NS60', 3='NS70', 4='NS80', 5='NS95', BEL07='Belgian coast 2007', THA07='Thames 2007', NOR07= 'Norfolk banks 2007'

Molecular analyses

DNA was extracted from the dried tissue covering the historical otoliths, using a protocol described in Cuveliers et al. (2009) and subsequently stored at -20°C. For the contemporary samples of 2007, DNA was extracted from fin tissue using the Nucleospin Tissue Purification kit (Macherey-Nagel GmBH). Both historical and contemporary samples were genotyped at 11 loci (F8-ICA9, F8-ITG11, F13-II8/4/7, (Iyengar et al. 2000); (Sos(AC)6, Sos(AC)20, Sos(AC)40, Sos(AC)30, Sos(AC)45 (Garoia et al. 2006); SolCA13, SolGA12 (Porta & Alvarez 2004); SseGATA26 (Funes et al. 2004) on an automated capillary sequencer ABI 3130 AVANT (Applied Biosystems). Microsatellite markers were combined in three multiplex-reactions. All PCR's were done using a touchdown protocol. Details on the PCR conditions are presented in the Supplementary Materials (Table S1). Allele size was determined using an internal lane size standard (250LIZ) and the software GENEMAPPER 3.7 (Applied Biosystems). The software TANDEM v. 1.07 was used for automated allele binning (Matschiner & Salzburger 2009). All recommendations for working with historical collections of fishes as suggested by Nielsen & Hansen (2008) were implemented. To avoid contamination, all sample extractions and PCR reactions were done in a fish DNA free laboratory with filter tips. Historical DNA was extracted under a laminar flow in a laboratory where no contemporary DNA work had been performed. Negative controls were included at all stages and approximately 20 % of all samples were re-amplified to check for reproducibility.

Data quality

The software MICROCHECKER 2.2.1 (van Oosterhout et al. 2006) was used to check for genotyping errors (null alleles, stuttering and large allele dropout). Null allele frequencies were estimated for each locus and population using the Expectation Maximization algorithm of Dempster et al. (1977), implemented in the software FREENA (Chapuis & Estoup 2007). All loci were tested for linkage disequilibrium with a permutation test in FSTAT v.2.9. (Goudet 2002). Deviations from Hardy-Weinberg equilibrium were tested by an exact test using a Markov Chain algorithm (Guo & Thompson 1992) in GENEPOP v. 4.0.10 (Raymond & Rousset 1995). Because N_e estimates assume purely genetic drift influences, we performed a neutrality test including all loci using the F_{ST} outlier method (*fdist*) of Beaumont and Nichols (1996), implemented in the software LOSITAN (Antao et al. 2008). A total of 95000 simulations were computed for SMM with the options 'neutral mean F_{ST}' and 'force mean F_{ST}'.

Spatial genetic variation

To avoid any bias on N_e estimates due to sampling location variation within the North Sea, we initially tested the genetic structure between geographically separated sampling locations. The level of spatial differentiation was first evaluated through comparison of the three samples from 2007, adjacent to three major spawning areas of the Norfolk Banks, the Belgian Coast and Thames estuary (Fig.1) (Rijnsdorp et al. 1992). Spatial differentiation was further tested by comparing historical samples within a sampling year from different locations. All tests were done with global and pairwise F_{ST} (θ) (Weir & Cockerham 1984) in GENETIX v. 4.05 (Belkhir et al. 2004) and Jost D estimate (Jost 2008) using the software SMOGD (Crawford 2010).

Temporal genetic variation

Given the large available dataset in this study, differences in genetic diversity over the sampling period were evaluated using three approaches. First, genetic diversity was compared *among sampling years*, consisting of several year classes. Secondly, genetic diversity was compared *among several cohorts* to analyse cohort-specific diversity. Only those cohorts with more than 28 individuals were retained. Finally, the 3-, 4-, 5- and 8-year old fish were selected because these *age groups* were composed of sufficient samples, and

the genetic diversity was compared among sampling years to investigate if genetic erosion has occurred due to differential fishing selection pressure on specific age groups.

To assess genetic diversity of each sample, observed (Ho) and unbiased expected (He) heterozygosity (Nei 1978) were calculated using GENETIX v. 4.05 (Belkhir et al. 2004). FSTAT v.2.9 (Goudet 2002) was used to calculate allelic richness (AR) (El Mousadik & Petit 1996) based on the smallest sample size for any locus. To test whether these measures of genetic diversity were significantly different among samples, they were compared for each locus with a non-parametric Friedman ANOVA (AR, He). Single and multilocus F_{IS} estimates (Weir & Cockerham 1984) were calculated in GENETIX v. 4.05. Multilocus F_{ST} estimates (Weir & Cockerham 1984) were also estimated as a measure of temporal differentiation among samples. Significance levels were assessed through 1000 permutations and corrected for multiple tests using a Bonferroni test (Rice 1989).

Effective population size

Estimates of effective population size were first calculated based on allele frequency differences among the temporal samples (mixed cohort design). There is currently no general consensus about which method performs best for larger populations (Fraser et al. 2007), hence we tested three methods to enable comparison to other (flat)fish studies (e.g. on plaice, Hoarau et al. (2005)). First, the temporal method of Jorde & Ryman (2007) was applied to estimate variance effective population size based on the unbiased estimator F_s'. This method generally performs better than other temporal methods if allele frequencies are skewed, a common feature in microsatellite data (Luikart et al. 1998; Jorde & Ryman 2007). Calculations were made using the software TempoFs following *"sample plan 2"* because individuals were sampled destructively before reproduction and were not returned to the population. In addition, N_e was calculated using the classical moment-based method of Waples (1989) and the pseudo-likelihood method of Wang (2001) (implemented in MNE v.1.0). The resulting long-term estimate (harmonic mean over the period 1957-2007) was compared with a temporal estimate over the first time interval (1950s-1960s) corresponding to a period with lower fishing pressure, and an estimate over the second time interval (1960s-2007) to explore temporal changes in effective population size in periods with an ever increasing fishing pressure. We also estimated N_e for each of the intermediate decades.

Because we do not have information on possible external source populations and because data from all sampling locations were pooled (see results section), the N_e estimate was based on the assumption of a closed population with N_e max set to 30 000 individuals. Finally, we compared N_e estimates based on temporal methods with single-sample estimates based on a linkage disequilibrium method (LDNE; (Waples & Do 2008)), which is considered an estimator of inbreeding N_e (Luikart et al. 2010). The method estimates N_e of the parental generation based on the correlation among alleles at unlinked loci and corrects for downward bias due to small sample sizes. Confidence intervals were determined by jackknifing on loci and the allowed frequency of observed alleles was set at 0.05 (Waples & Do 2008) because alleles occurring at low frequencies may bias results (Waples 2006).

An important changing demographic parameter in harvested fish populations is the generation time (Tg), defined as the mean age of the parents (Hill 1979). This parameter is generally overlooked in other studies assessing effective population size, most likely resulting in an underestimation of population size due to an unaccounted increase in generation number between recent time intervals. Here, we corrected for such bias by calculating generation time using the estimates of the von Bertalannfy growth parameters, weight-length relationship, maturity – age relationship estimated from fish sampled during research vessel surveys and from landings of commercial fisheries (Mollet et al. 2007), relative fecundity estimates (Witthames et al. 1995), natural and fishing mortality (ICES 2009). In sole, Tg has strongly decreased over the sampling period from 7.7 years in the 1950s to 4.2 years in the 1990s (Fig. 2). Because this is important information when estimating the number of generations between temporal samples, the change in generation time was taken into account in the analyses by altering the generation time each decade according to Fig.2. The mean estimated generation length over the whole study period was 4.9 years (average male and female) and the estimated number of generations between the first and last temporal sample was 10. The estimated number of generations during the first time interval (NS50-NS60) was only one generation, while the estimated number of generations for the second time interval (NS60-NS07) was 9 generations (Table 3).

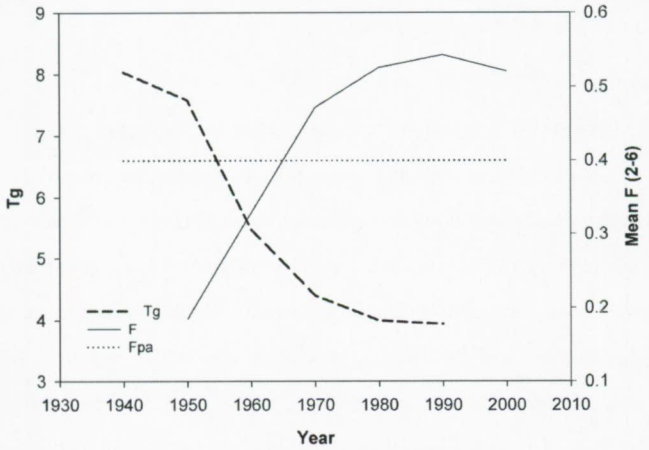

Fig. 2 *Solea solea.* Evolution of fishing mortality F (age 2-6) and generation time (Tg) (average of male and female, in years) in the North Sea (ICES IVc) from 1940 to 2000. Fpa: Precautionary approach fishing mortality

Estimates of N_e/N_c ratio

The census population size was estimated for the different time periods as the mean of the population numbers (age 3 to 10), as estimated by stock assessments (ICES 2009). The ratio N_e/N_c was then calculated using the results of the N_e estimations for the corresponding time intervals.

Power analysis

We evaluated the statistical power to detect genetic differentiation with POWSIM (Ryman & Palm 2006). The program simulates samples from multiple populations that have drifted apart a number of generations and calculates the expected F_{ST} and *P*-values for a given N_e assuming no mutation or migration. The null hypothesis of genetic homogeneity is tested with a Fisher exact test and a chi-square test. The proportion of significances ($P < 0.05$) represents an estimate of the power while the error rate of rejecting H_O when it is true (type I error) is calculated using samples drawn directly from the base population without the drift (Ryman & Palm 2006). Power was assessed across 200 runs of 10^3 iterations each. To test the amount of drift that would be detectable with the number of loci ($L = 11$) and the sample size ($N = 250$) of our study, several simulations were done with varying N_e values and ten generations of drift. Next, simulations were performed to evaluate the number of loci and sample size necessary to detect population differences with an effective population size similar to the one observed in our study ($N_e = 2000$).

RESULTS

Data quality

We found no evidence for large-allele dropout with MICROCHECKER. Estimated null allele frequency per locus calculated with the algorithm of Dempster ranged from 0 to 0.28. MICROCHECKER and FREENA found evidence for null alleles at three loci: Sos(AC)30, Sos(AC)40 and Sos(AC)45. Therefore, all analyses were done with and without these loci. Because the exclusion of these loci did not have any significant influence on the outcome of the diversity analyses or N_e estimates and because a jack-knife procedure did not indicate a large influence from any of these loci on temporal F_{ST}, we decided to keep the markers in the analyses in order not to lose statistical power. The test with LOSITAN confirmed selective neutrality for all microsatellite loci. The historical DNA was of sufficiently high quality to result in a PCR re-amplification success, ranging from 91 % to 97 %. Linkage equilibrium was met in all pairwise loci combinations after 1320 permutations.

Spatial variation

The global F_{ST} for the recent samples of 2007 did not point to any spatial differentiation (F_{ST} = -0.0006; p = 0.639; 95 % CI: -0.00203 - 0.00110). None of the pairwise F_{ST} values were significantly different from zero (p > 0.59). Jost *D* estimate was also very small (D_{est} = 0.001) and all pairwise *D* estimates were equal to 0.001. In the following temporal analyses, the three samples were therefore pooled ('NS07'). The geographical analysis of historical samples sampled in the same year also showed that there were no significant pairwise genetic differences among samples from the southern North Sea (F_{ST} ranging from -0.007 to 0.005, p > 0.083), indicating the lack of small scale spatial influence on allele frequencies. Additionally, because the samples from the different sampling years were homogenously distributed across the study area, no confounding effects of geographic differentiation were expected. Genotypes across sampling locations were thus pooled by sampling year in order to provide a global N_e estimate for the North Sea.

Genetic diversity

-Comparison of sampling years- Several loci and samples showed significant deviation from Hardy-Weinberg equilibrium (significantly higher F_{IS} than expected by chance) after Bonferroni correction (Supplementary Materials, Table S3). The highest prevalence of

heterozygote deficiencies occurred at locus Sos(AC)30 and Sos(AC)40. Multilocus F_{IS} values ranged from 0.09 (NS80) to 0.16 (NS60) and were all significant (Table 1). All loci were polymorphic with the number of alleles sampled ranging from 5 (Locus F8-ICA9) to 34 (Locus Sos(AC)20)). There were no private alleles with a frequency > 0.02. There were no indications of temporal change in genetic diversity (allelic richness or expected heterozygosity) over the sampling period (Table 1). Average allelic richness based on 88 individuals was similar in all temporal samples (ANOVA χ^2 (N = 11, df = 5) = 4.766, p = 0.445). Unbiased expected heterozygosity per locus ranged from 0.46 to 0.90 (Table S3). Mean expected heterozygosity was 0.77 for all the historical samples (NS50-NS60-NS70-NS80-NS95) and was slightly higher in the contemporary samples (NS07: He = 0.78), but this difference was not significant (χ^2(N = 11, df = 5) = 5.706, p = 0.336) (Table 1).

Table 1 Summary statistics of genetic variability by sampling year and by cohort. Abbreviations: Number of samples (N), Expected heterozygosity (He), observed heterozygosity (Ho), allelic richness (AR) and FIS values over all loci. Significant values are in bold. AR (sampling year) based on 88 individuals, AR (cohort) based on 20 individuals.

		N	He	Ho	AR	F_{IS}
SAMPLING YEAR	NS50	215	0.773	0.673	13.92	**0.130**
	NS60	205	0.774	0.648	13.16	**0.163**
	NS70	205	0.774	0.674	14.15	**0.128**
	NS80	404	0.775	0.705	13.93	**0.090**
	NS95	130	0.768	0.669	13.90	**0.129**
	NS07	220	0.782	0.689	13.78	**0.118**
COHORT	1947	38	0.776	0.685	8.89	**0.117**
	1950	30	0.787	0.644	9.48	**0.182**
	1953	33	0.768	0.687	8.75	**0.105**
	1954	41	0.761	0.680	9.27	**0.107**
	1958	45	0.777	0.648	9.46	**0.166**
	1963	120	0.780	0.661	9.31	**0.152**
	1967	28	0.769	0.676	8.98	**0.122**
	1969	67	0.779	0.667	9.56	**0.144**
	1979	62	0.759	0.708	9.11	**0.067**
	1980	67	0.774	0.704	9.15	**0.091**
	1981	148	0.778	0.705	9.52	**0.093**
	1984	29	0.778	0.722	9.35	**0.072**
	1991	77	0.767	0.680	8.99	**0.114**

Multilocus F_{ST} using the whole dataset consisting of both historical and contemporary samples was 0.0005 (95 % CI: 0.0000 - 0.0012) and not significantly different from zero (p = 0.290). Estimates of temporal genetic differentiation based only on the historical samples was F_{ST} = 0.0003 (95 % CI: -0.00011 - 0.00090) and not significantly different from zero (p = 0.112).

-Comparison of cohorts- Genetic diversity did not differ significantly among cohorts (Table 1). Allelic richness based on 20 individuals varied from 8.7 (cohort 1953) to 9.5 (cohort 1969), but showed no significant differences among cohorts (ANOVA χ^2(N = 11, df = 12) = 15.704, p = 0.205). Expected heterozygosity was almost constant for all cohorts (ranging from 0.759 to 0.787) (ANOVA χ^2(N = 11, df = 12) = 12.979, p = 0.371). Multilocus F_{IS} values ranged from 0.07 (cohort 1979) to 0.18 (cohort 1950) and were almost all significant (Table 1). We could not detect any temporal differentiation among cohorts, with a multilocus F_{ST} of 0.0003 (95 % CI: -0.00041 - 0.00099) which differed not from zero (p = 0.299).

-Comparison of age groups- Individuals were also clustered according to their age (per sampling year) to test for changes in genetic diversity due to differential fishing selection pressure on specific age groups. Allelic richness based on 13 individuals ranged from 7.43 to 8.05 but was not significantly different among age groups (age 3, 4, 5, 8) (χ^2(N = 11, df = 15) = 8.948, p = 0.880). Expected heterozygosity ranged from 0.759 to 0.782 without any significant differences among age groups (χ^2(N = 11, df = 15) = 10.788, p = 0.767) (Table 2). There was no differentiation among samples; multilocus F_{ST} was 0.00007 (95% CI: -0.00119-0.00110; p = 0.458).

Table 2 Summary statistics of genetic variability by age, for 3, 4, 5 and 8 year old fish clustered by sampling year. He: expected unbiased heterozygosity; Ho: observed heterozygosity; AR: Allellic Richness based on 13 diploid individuals (NA = not available due to insufficient samples); F_{IS}: Weir & Cockerham's (1984) estimate of fixation index. Significant F_{IS} values are in bold.

Sampling year	N	Age	He	Ho	AR	F_{IS}
NS50	26	3	0.771	0.685	7.48	**0.114**
NS50	45	4	0.766	0.687	7.65	**0.105**
NS50	21	5	0.761	0.674	7.51	**0.118**
NS50	22	8	0.782	0.648	7.64	**0.176**
NS60	58	3	0.778	0.627	7.51	**0.196**
NS60	46	4	0.777	0.689	7.66	**0.115**
NS60	30	8	0.780	0.650	7.80	**0.170**
NS70	73	3	0.772	0.660	7.74	**0.147**
NS70	33	4	0.776	0.655	7.43	**0.159**
NS70	30	5	0.759	0.685	7.55	**0.100**
NS70	10	8	0.764	0.736	NA	0.041
NS80	143	3	0.782	0.727	8.05	**0.070**
NS80	87	4	0.778	0.712	7.82	**0.085**
NS80	72	5	0.762	0.696	7.73	**0.087**
NS80	24	8	0.766	0.697	7.59	**0.092**
NS95	11	3	0.779	0.614	NA	**0.221**
NS95	76	4	0.768	0.677	7.55	**0.118**
NS95	22	8	0.774	0.686	7.68	**0.117**

Effective population size

Estimates of long-term effective population size using temporal methods are listed in Table 3. Overall, there was great congruence between the N_e estimates for the three temporal methods. Estimation of the harmonic mean N_e over the whole period from 1957 (NS50) to 2007 (NS07) using the moment-based method of Waples (1989) was 2247 with a finite confidence interval (CI = 1126-8370). The pseudo-likelihood method of Wang (2001) resulted also in an estimate larger than 2000 (N_e = 2169) with a finite confidence interval (CI = 1221-5744). The method of Jorde and Ryman (2007) yielded an estimate of N_e = 4394, but with an upper confidence interval reaching infinity.

The N_e estimate for the first time interval (NS50-NS60) was an order of magnitude lower than for the period after the 1960s (NS60-NS07), although the upper confidence limits

ranged to infinity (Table 3). Lower confidence limits were very low and ranged from 80 (Jorde & Ryman 2007) to 319 (Wang 2001) for the first interval.

N_e estimates for the second period (NS60-NS07) was 1456 (Waples 1989), 2054 (Wang 2001) and infinity (Jorde & Ryman 2007). Both the temporal method of Waples (1989) and the pseudolikelihood of Wang (2001) resulted in finite upper confidence limits exceeding 3000. The lower confidence limits for this time interval ranged from 799 to 2378. Allele frequency changes (Fs') were smaller and thus the N_e estimates were higher for the intermediate time intervals with an increasing number of generations between the temporal points (Table 3).

Single sample estimates of N_e (LDNE) for each of the sampling years separately were always larger than 1000 with very broad confidence intervals. Upper confidence limits were infinitely large in all years, lower confidence limits ranged from 289 in NS50 to 1826 in NS70 (Table 4).

Estimates of N_e/N_c ratio

The mean census size N_c for the North Sea stock for the whole period (1957-2007) was estimated at 200.10^6 individuals (Table 3). The average census population size decreased from 341.10^6 in the first time interval (1950-1960) to 145.10^6 in the last decade (1995-2007). With an estimate of N_e around 2000, this results in an overall ratio N_e/N_c of 10^{-5}.

Table 3 Temporal estimates of effective population size N_e [95 % C.I.], for the different time intervals using 3 methods, with corresponding generation time (Tg) and number of generations between sampling points (G). Measure of allele frequency change (Fs') with standard error (S.E.) following (Jorde & Ryman 2007). Estimates of census population size (N_c) and N_e/N_c are also given.

Interval	Tg	G	Fs' (S.E.)	Jorde & Ryman 2007		Waples 1989		Wang 2001		N_c	N_e/N_c
NS50-NS60	6.3	1	0.0014 (0.0024)	346	[80- ∞]	458.4	[195.1 -84598.5]	669	[319-∞]	341×10^6	10^{-6}
NS60-NS70	4.9	1	0.0010 (0.0022)	467	[92-∞]	523.8	[206.6-∞]	2180.9	[511.8-∞]	207×10^6	10^{-6}
NS70-NS80	4.3	3	0.0004 (0.0017)	4398	[406-∞]	2506	[915-∞]	2576	[1154-∞]	126×10^6	10^{-5}
NS80-NS95	4.2	2	0.0004 (0.0016)	2359	[286-∞]	987.9	[396.6-∞]	843.9	[449.7-3870.2]	222×10^6	10^{-6}
NS95-NS07	4.2	3	-0.0004 (0.0014)	-4219	[619-∞]	2904	[669.7-∞]	843.9	[449.7-3870.2]	145×10^6	10^{-5}
NS60-NS07	4.5	9	-0.0004 (0.0012)	-10726	[2378-∞]	1456	[799.8 - 3667.1]	2054	[1133.5 - 6235.7]	173×10^6	10^{-5}
NS50-NS07	4.9	10	0.0011 (0.0015)	4394	[1256 - ∞]	2247	[1126.8 - 8370.1]	2168.8	[1221.4 - 5743.5]	200×10^6	10^{-5}

Table 4 Single sample estimates of effective population size [95 % C.I.] for each of the temporal samples, based on the Linkage disequilibrium method (Waples & Do 2008). N = sample size.

	Sampling year					
	NS50	**NS60**	**NS70**	**NS80**	**NS95**	**NS07**
	N = 215	N = 205	N = 205	N = 404	N = 130	N = 220
LDNE	1647.9	2333.4	INF	4342.5	INF	INF
(Waples & Do 2009)	[289.3 INF]	[367.1 INF]	[1826.3 INF]	[629.1 INF]	[441.1 INF]	[611.6 INF]

Power analysis

The power analysis indicated that the tests provided a sufficient amount of statistical power to detect the level of genetic differentiation observed, since a true F_{ST} of 0.001 (N_e = 5000) would be detected with a probability of 98 % with the observed allele frequencies at the 11 loci and with a sample size of 250 (Fig. 3).

The simulations further showed that a sample size of 80 was enough to obtain 88% probability of detecting genetic differences with N_e = 2000 and 11 loci. There was no strong effect of the number of loci with an effective population size of 2000 and a sample size of 250.

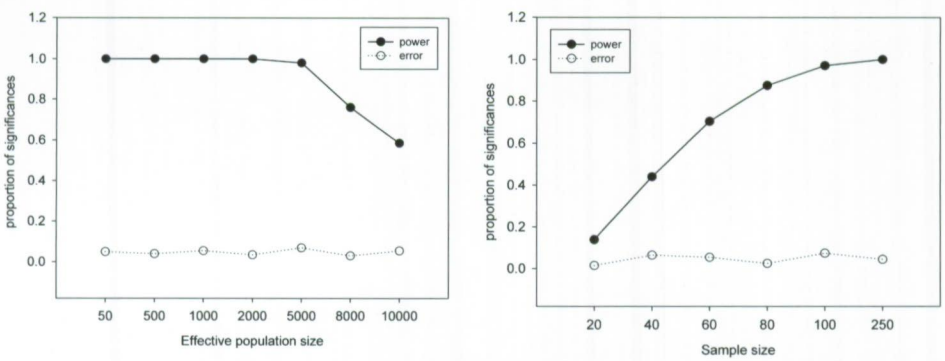

Fig. 3 Simulated estimates (average of 200 runs) of power (solid line) and α-error (stippled line). Left figure presents results for the present set of microsatellite loci (L = 11), sample size (N = 250) and alleles at different effective population sizes. Right figure presents results for a fixed N_e = 2000, fixed number of loci (L = 11), and different sample sizes. See text for details.

DISCUSSION

Changes in life history traits have been observed in *Solea solea* in the North Sea over the last decade. The rapid decrease in age and size at first maturation has been linked to increasing fishing pressure (Mollet et al. 2007). Since the end of the 1960s, fishing mortality has always exceeded the precautionary approach mortality of F = 0.4. In this study we therefore tested the hypothesis that such continuous increase in fishing intensity should also be reflected in a decrease in neutral genetic diversity of the stock and hence a reduction in effective population size.

Despite the decrease of spawning stock biomass of more than 50 % since the late 1950s (ICES 2009) and the above mentioned indications for rapid phenotypic changes over ten generations of heavy fishing (50 years), we could not observe a long-term decrease in neutral genetic diversity (neither in allelic richness nor expected heterozygosity) in North Sea sole between 1957 and 2007. In age structured populations with overlapping generations, allele frequency fluctuations might occur due to the unequal participation of cohorts to reproduction. All cohorts in this study showed however similar levels of genetic diversity and the analyses failed to identify such genetic differences between year classes. There were also only slight differences in genetic diversity between age groups; such differences could arise as a result from the gradual extirpation of genetic diversity due to selective harvesting of larger (and older) individuals. We could not find indications to support this hypothesis based on our data.

Our results are in line with various other studies on heavily exploited marine fish also showing a stable temporal pattern of neutral diversity (Ruzzante 2001; Poulsen et al. 2006; Chevolot et al. 2008; Larsson et al. 2010; Therkildsen et al. 2010). Our data included samples from the 1950s, corresponding to a period of lower fishing mortality. Despite the different approaches used to explore temporal diversity, none of them indicated a change in genetic composition. Such results form one of the main challenges in monitoring genetic changes in marine heavily exploited species composed of large open populations. In contrast to observed life-history traits shifts (selection acts much faster than drift in large populations) and diminishing census sizes, neutral diversity is most likely only lost substantially when

marine populations are both very small and isolated (Nm < 1 %) (Palstra & Ruzzante 2008). Inbreeding, recessive alleles and reduced adaptive potential then tend to speed up the extinction process. For marine species, the fishery would likely collapse economically before the population decreases down to the level when genetic drift becomes detectable (Boehlert 1996). The absence of changes in neutral genetic variation could simply be the consequence of the large effective population size, acting against the effects of genetic drift.

In line with a stable genetic diversity, the estimated N_e of sole for the period 1957 – 2007 was higher than 2000 individuals, with lower confidence boundaries higher than 1000. The N_e did not seem to decrease over the 50-year time period, regardless of the temporal methods used. Several of the estimated confidence intervals reached infinity. The broad confidence intervals show that, in species with high population sizes, it is difficult to distinguish between moderate and high N_e with many methods. This is because the sampling variance (1/(sample size)) is much larger than the genetic drift per generation ($1/(2N_e)$), resulting in a large noise-to-signal ratio (Poulsen et al. 2006; Luikart et al. 2010). Single-sample estimates all showed very broad confidence intervals, ranging from 289 to infinity. Lower confidence limits were lowest in the 1950s, maximal in the 1970s and decreased again after the 1970s, although the upper limits could never be distinguished from infinity. Linkage disequilibrium methods seem to perform poorly with large population sizes (> 10000) such as in sole (Tallmon et al. 2010).

The observed N_e estimate in sole suggests that on the one hand genetic drift is limited and did not really influence allele frequencies over the last 50 years or alternatively, that gene flow levels from neighbouring populations (such as the Bay of Biscay, English Channel, Skagerrak) are so high to buffer for any loss of genetic variation. A combination of both processes is also possible. In theory, estimates of $N_e > 1000$ are sufficiently high to avoid inbreeding depression and to maintain evolutionary potential (Frankham et al. 2002), although these 'rules of thumb' should be regarded as rough guidelines and some authors have argued that N_e values should be higher than 5000 to maintain evolutionary potential (Franklin & Frankham 1998). For a marine fish with highly variable reproductive success, an effective size of 2000 might be considered rather low. Moreover, the lower confidence boundaries of the estimates were in several instances lower than 1000.

Compared to the census estimates of sole (millions of fish), the obtained N_e estimates are indeed relatively small. This is also reflected in the low N_e/N_c ratio (10^{-5}) for sole. A similar estimate of N_e/N_c was obtained for plaice, a flatfish mainly caught together with sole in a mixed fishery in the North Sea (Hoarau et al. 2005), but also for North Sea cod, the ratio was estimated at 10^{-5} (Hutchinson et al. 2003). Small N_e/N_c ratios are commonly observed in marine fish (Hauser et al. 2002; Poulsen et al. 2006; Chevolot et al. 2008; Larsson et al. 2010) and are mainly caused by the fluctuating population sizes, unequal sex ratio and the high variance in reproductive success resulting from a type III survivorship (Palstra & Ruzzante 2008). In North Sea sole, fluctuations in stock size are observed over the study period, caused by the occurrence of very strong and very weak year classes (Rijnsdorp et al. 1992; ICES 2010). Such fluctuations might decrease the N_e, but are less important for short term estimates of N_e (across a few generations), unless a bottleneck has occurred (Hauser & Carvalho 2008). There is no evidence for biased sex ratio in North Sea sole. As in most marine fish, the primary factor causing the low N_e/N_c ratio in sole is most likely the large variability in reproductive success arising from variance in family reproductive success and variance in individual lifetime reproductive success. Fecundity in sole ranges from 200 000 – 450 000 eggs for a female of 35 cm (Witthames et al. 1995) and larval mortality rates range from 10 – 50 % per day (Horwood 2001). The combination of high fecundity and high mortalities early in life may result in sweepstakes recruitment, where most of the progeny are only from very few parents (Hedgecock 1994). Hence, high variance in individual reproductive success is likely.

Traditionally, it is assumed that a positive linear relation exists between the census size and effective size, leading to a stable N_e/N_c ratio for species (Frankham 1995b). Although such a constant ratio would have a practical value in conservation, in many species the N_e/N_c ratio is not necessarily fixed and the relationship between N_e and N_c is better explained by a power function (Palstra & Ruzzante 2008). This means that the ratio might even increase with decreasing census sizes. Such an increase in N_e/N_c ratio was also observed for sole in our study; several hypotheses can be put forward to explain this. First, there may exist a mechanism of genetic compensation acting against the negative genetic effects of low population numbers (Palstra & Ruzzante 2008). Such a density-dependent relationship

between N_e and N_c has been illustrated in empirical studies of fish and might be explained by the reduction of competition for mating partners or spawning sites at lower census sizes (Ardren & Kapuscinski 2003; Araki et al. 2007). Second, the reproductive success in marine fish depends strongly on the larger and older females, producing more eggs (Chambers & Leggett 1996; Palumbi 2004a; Wright & Trippel 2009); this is also true for sole (Horwood 2001; Rijnsdorp & Witthames 2004). The egg weight and size is also proportional to female size or age in several marine fish, with potential effects on larval viability (Rijnsdorp & Vingerhoed 1994; Berkeley et al. 2004; Higashitani et al. 2007). The removal of those older and larger females by a size-selective fishery might give the many other, less productive females the opportunity of becoming 'reproductive winners'. This might in its turn reduce the variance in reproductive success among families/individuals, increasing the N_e and as such also the ratio N_e/N_c.

Besides the biological explanations for the observed low N_e/N_c in sole, caution is advised about potential violation of assumptions underlying the N_e methods, inducing bias. The temporal methods applied here are developed for species with non-overlapping generations and assume negligible immigration from other populations. For species with a type III survival such as sole, bias due to overlapping generations would lead to underestimate N_e (Waples & Yokota 2007). Although the assumption of discrete generations is violated for sole, the longest time intervals (1957-2007) in this study cover > 9 generations, minimizing the bias due to overlapping generations (Waples & Yokota 2007). For the N_e estimates over the shortest time intervals separated by only one generation, age structure could be responsible for the lower values, although the upper confidence limits were infinitely large. We assumed North Sea sole to be a single (meta)population and that all temporal samples were taken from this population. Based on neutral genetic markers, there are no indications for population substructure at this spatial scale (Kotoulas et al. 1995; Exadactylos et al. 1998; Rolland et al. 2007) (Chapter 1). The assumption of no migration from outside the study area might have been violated in our study if gene flow (e.g. from the English Channel) actually did occur during the sampling period. An upward bias is expected if migration occurs from a genetically similar source, counteracting the effects of drift (Fraser et al. 2007). This is the most likely scenario for sole, because no genetic differences were found between sole from the English Channel and the North Sea (Chapter 1).

Finally, technical errors due to mis-scoring or the presence of null alleles remain a concern in historical samples. Nevertheless, the analysis of N_e without the loci with high null allele frequencies yielded similar estimates. This was also shown in a study of Turner et al. (2002), indicating that N_e estimates were robust for the presence of null alleles. To exclude the possibility that we could not detect the effects of genetic drift due to limited power, simulations were performed. The simulations showed that strong genetic differentiation due to genetic drift alone was detectable with the number of loci and sample size comparable to our study. Furthermore, with an effective population size of 2000 (as estimated in our study), the number of loci did not greatly impact the differentiation power. Hauser & Carvalho (2008) described that, under complete isolation and with an effective population size between 10^3 and 10^4 as observed in our study, only 10 generations are required to achieve an F_{ST} of 0.002.

Even though there was no apparent loss in neutral genetic diversity, we must be cautious on concluding the absence of fishery effects on genetic diversity. Because of the low correlation between neutral diversity and quantitative variation (Reed & Frankham 2001), the possibility that there has been a decrease in adaptive genetic variation cannot be excluded. If the population is indeed large, it might be prone to the rapid effects of selection (natural or human-induced). For now, we can only conclude that the population size reduction due to overfishing has not yet reached the level to increase genetic drift drastically (in the absence of gene flow).

To conclude, our results do not point to any measurable changes in the level of neutral genetic diversity for *Solea solea* over the time period between 1957 to 2007, corresponding to a period with a major increase in fishing intensity. The effective population size for sole is most likely larger than 2000, suggesting that genetic drift is not of major importance in this population. The current stable neutral variation together with life-history trait data will serve as a perfect baseline dataset to pick-up even slight footprints of selective anthropogenic pressure and to highlight the evolutionary consequences at the genomic level of overharvesting by humans.

ACKNOWLEDGEMENTS

Research was funded by the European Community's sixth Framework Programme under contract no. FP6-044276 (FinE). The authors thank S. Geldof and C. Vanderheydt (Katholieke Universiteit Leuven) for their help with the laboratory work. Many thanks to I. Pennock, L.J Bolle, F.M. Mollet from IMARES for the exchange of the otolith samples and for providing us with the corresponding age and length data. We also thank R. Waples, J. Wang and P.E. Jorde for their helpful comments or information. E.C. acknowledges a PhD grant from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). G.E.M. is a post-doctoral researcher funded by the Research Foundation-Flanders (FWO-Vlaanderen). M.H.D.L. received a postdoctoral position of the K.U.Leuven (BOF PDM-Kort). Three anonymous reviewers are greatly acknowledged for useful comments.

SUPPLEMENTARY MATERIALS

Table S1. Multiplex conditions for *Solea solea*: primer concentration and annealing temperature.

Multiplex	Locus	GenBank Accession No.	Conc (µM)	Ta	
1	Sos(AC)40	AY950592.1	0.1		
1	Sos(AC)45	AY950593.1	0.1		
1	Sos(AC)30	AY950589.1	0.1	59°C to 56°C	with -1°/cycle: 4 x
1	SolCA13	AF441390.1	0.1	56°C: 34 x	
1	Sos(AC)20	AY950591.1	0.1		
2	F13-II8/4/7	AF173849.1	0.4		
2	SolGA12	AF441391.1	0.1	67°C to 57°C	with -1°C/cycle: 11x
2	F8-ITG11	AF173855.1	0.1	57°C: 29 x	
2	Sos(AC)6	AY950588.1	0.4		
3	F8-ICA9	AF173851.1	0.2	60°C to 54°C	with -1°C/cycle: 7x
3	SseGATA26	AB177537.1	0.2	54°C: 29 x	

PCRs were carried out in 10 µL volumes, using a Multiplex PCR Kit (Qiagen), forward (fluorescently labeled) and reverse primers and 1 µL of template DNA. All PCRs followed a touchdown protocol. PCR conditions were as follows: 95 °C for 15 min, 95 °C for 30 s, Ta (Table) for 90 s, 72 °C for 60 s, 95 °C for 30 s, Ta (Table) for 90 s, 72 °C for 60 s, 60 °C for 30 min followed by storage at 4 °C.

Table S2 Age composition (numbers of individuals) by sampling years. Sampling years are grouped in decades, labeled as 'NS50', 'NS60', 'NS70', 'NS80', 'NS95', 'NS07'.

	SAMPLING YEAR														
	NS50			NS60		NS70				NS80			NS95	NS07		
AGE	**1957**	**1958**	**1959**	**1966**	**1967**	**1971**	**1972**	**1973**	**1974**	**1984**	**1985**	**1987**	**1995**	**BEL07**	**NOR07**	**THA07**
2		3				1	1	1	1	2				7	13	
3	7	13	6	43	15	8	54	3	8	112	4	27	11	2	10	10
4	14	22	9	1	45	6	13	8	6	49	11	27	76		7	5
5		14	7	1	1	6	20		4	45	7	20	3		5	2
6	3	5	2	14		2	5	1	1	2	6	24	7	1	5	
7	6	18		7	5	1	3		3	11		11	2		3	
8	3	16	3	28	2	3	6	1		13	2	9	22		1	
9	2	12	4	6	9	1	16			4	3		4		1	
10	8	1	4	5				4		1		2	2			
11	1	24		2		2			4	2	1	2	2			
12		1	2	3	1		2									
13				2	2											
14	1	1			1		5	1								
15		1			1			1				1				
16				2					1							
17				2	1				1							
19				5						2			1			
21		1								3						
22		1														
25										1						
NA					1		1							77		71
Total	45	133	37	121	84	30	126	20	29	247	34	123	130	87	45	88
Total Decade		215		205			205				404		130		220	

Table S3 Summary statistics per locus by sampling year. For sample codes, see main text. N = Number of samples; AR = allellic richness (based on 43 individuals); Na = number of alleles; Ho = observed heterozygosity; He = unbiased expected heterozygosity; f = fixation index; HW = p-value corresponding to Hardy-Weinberg Exact Test (Genepop)

Locus	NS50	NS60	NS70	NS80	NS95	BEL07	NOR07	THA07	Locus	NS50	NS60	NS70	NS80	NS95	BEL07	NOR07	THA07
SolCA13									F13								
N	198	194	196	388	124	87	45	88	N	211	200	203	401	130	87	45	88
AR	9.65	9.05	9.47	9.67	8.79	9.54	9.91	9.26	AR	10.69	11.01	9.60	10.97	10.05	11.02	8.87	12.06
Na	12	11	11	13	9	11	10	10	Na	15	15	13	16	13	14	9	16
He	0.814	0.816	0.824	0.830	0.810	0.812	0.812	0.819	He	0.732	0.748	0.704	0.735	0.721	0.752	0.704	0.745
Ho	0.773	0.763	0.770	0.814	0.758	0.793	0.733	0.761	Ho	0.649	0.625	0.640	0.696	0.700	0.644	0.600	0.727
f	0.05	0.066	0.065	0.019	0.064	0.024	0.097	0.071	f	0.113	**0.165**	0.091	0.054	0.03	0.145	0.149	0.024
HW	0.065	0.087	0.0209	0.8768	0.1322	0.6609	0.3352	0.1815	HW	0.0176	0.0238	0.0194	0.0602	0.1199	0.0651	0.137	0.3498
Sos(AC)20									F8-ITG11								
N	213	205	203	400	130	87	45	88	N	201	199	162	292	88	87	45	88
AR	17.80	18.82	18.74	18.72	18.43	18.97	16.73	17.38	AR	9.64	8.75	11.12	10.03	7.97	9.96	10.87	9.99
Na	27	28	27	34	28	25	17	24	Na	14	10	15	13	9	12	11	11
He	0.806	0.814	0.827	0.805	0.797	0.813	0.845	0.797	He	0.783	0.778	0.793	0.802	0.762	0.800	0.821	0.798
Ho	0.765	0.771	0.778	0.760	0.785	0.816	0.822	0.727	Ho	0.781	0.759	0.827	0.822	0.716	0.759	0.800	0.807
f	0.05	0.053	0.059	0.056	0.016	-0.004	0.027	0.087	f	0.002	0.025	-0.044	-0.025	0.06	0.052	0.026	-0.011
HW	0.0606	0.0688	0.0745	0.0121	0.8669	0.5175	0.6171	0.0071	HW	0.2083	0.0033	0.0711	0.001	0.063	0.0197	0.1283	0.2403
Sos(AC)30									SolGA12								
N	214	204	200	389	129	87	45	75	N	200	203	184	378	126	87	45	88
AR	12.09	12.43	12.96	11.88	11.98	12.18	11.91	11.29	AR	13.34	13.22	14.12	14.99	13.66	13.38	10.91	11.29
Na	16	17	17	16	15	15	12	13	Na	19	17	20	22	18	17	11	13
He	0.833	0.815	0.828	0.809	0.795	0.831	0.791	0.834	He	0.820	0.821	0.841	0.840	0.834	0.811	0.801	0.798
Ho	0.664	0.662	0.635	0.663	0.620	0.632	0.511	0.613	Ho	0.825	0.803	0.821	0.839	0.810	0.782	0.778	0.750
f	**0.20**	**0.19**	**0.23**	**0.18**	**0.22**	**0.24**	**0.36**	**0.27**	f	-0.006	0.022	0.024	0.001	0.029	0.037	0.029	0.061
HW	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	HW	0.0783	0.1684	0.1807	0.0081	0.368	0.4911	0.324	0.3383
Sos(AC)40									Sos(AC)6								
N	167	151	165	359	107	19	45	78	N	201	196	199	399	129	87	45	88
AR (19)	11.76	9.63	10.86	10.69	10.36	9.00	9.99	9.27	AR	13.68	14.32	14.35	13.86	13.89	13.91	15.78	12.69
Na	18	11	15	16	17	9	12	13	Na	19	19	22	23	18	15	16	14
He	0.898	0.880	0.888	0.883	0.864	0.875	0.869	0.860	He	0.885	0.892	0.880	0.886	0.880	0.885	0.884	0.883
Ho	0.569	0.351	0.503	0.593	0.421	0.579	0.556	0.449	Ho	0.746	0.745	0.749	0.792	0.760	0.701	0.756	0.750
f	**0.37**	**0.60**	**0.43**	**0.33**	**0.51**	**0.34**	**0.36**	**0.48**	f	**0.16**	**0.17**	**0.15**	**0.11**	**0.14**	**0.21**	0.15	**0.15**
HW	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	HW	**0.000**	**0.0009**	**0.0001**	**0.000**	0.1471	0.0036	0.0486	0.0112
Sos(AC)45									SseGATA26								
N	182	199	198	397	129	86	45	88	N	198	196	191	374	116	86	43	88
AR	11.47	10.65	11.11	11.47	11.93	10.49	9.95	12.06	AR	10.11	9.27	10.34	9.99	9.97	11.07	13.00	11.64
Na	15	14	15	17	15	13	10	14	Na	19	19	17	21	15	14	13	15
He	0.864	0.868	0.875	0.873	0.874	0.863	0.869	0.878	He	0.576	0.561	0.588	0.599	0.617	0.682	0.741	0.686
Ho	0.665	0.653	0.677	0.761	0.767	0.826	0.733	0.818	Ho	0.551	0.526	0.534	0.559	0.586	0.674	0.698	0.671
f	**0.23**	**0.25**	**0.23**	**0.13**	**0.12**	0.04	0.16	0.07	f	0.04	0.06	0.09	0.07	0.05	0.01	0.06	0.02
HW	**0.000**	**0.000**	**0.000**	**0.000**	0.4847	0.0625	0.3244	0.0642	HW	0.0068	0.81	0.0041	0.3758	0.3247	0.1629	0.0968	0.3417
F8-ICA9																	
N	207	192	187	369	129	87	45	88									
AR	4.60	4.54	4.77	4.39	5.41	4.24	5.87	4.97									
Na	7	7	8	9	8	5	6	6									
He	0.498	0.518	0.462	0.465	0.494	0.476	0.507	0.497									
Ho	0.416	0.469	0.481	0.461	0.434	0.529	0.511	0.477									
f	0.165	0.095	-0.042	0.009	0.121	-0.112	-0.008	0.04									
HW	0.0024	**0.000**	0.0943	0.2186	0.0066	0.7462	0.506	0.1133									

GENERAL DISCUSSION

Today, over 75 % of the monitored marine fish stocks are fully exploited or overexploited (OSPAR 2010). The conventional wisdom that the large size of marine fish populations will protect them from losing genetic diversity has been repeatedly challenged (Hauser et al. 2002; Kenchington et al. 2003). Even worse, the evidence that fisheries-induced selection is causing genetic changes in fish populations is growing (Grift et al. 2003; Jørgensen et al. 2007; Law 2007; Mollet et al. 2007; Dunlop et al. 2009).

In order to preserve (genetic) diversity and develop management strategies that may help stocks to recover from overexploitation, knowledge on the population connectivity and migration patterns of juvenile and adult fish is essential. Due to the open nature of marine systems and due to their specific life history characteristics, marine populations are thought to be well connected (DeWoody & Avise 2000). However, recent studies demonstrating fine scale population structure in 'classical marine fishes' challenge this traditional view (Hauser & Carvalho 2008) and often point to a mismatch between the scale of biological stock structure and the scale of fisheries management (Reiss et al. 2009).

For analyzing changes in genetic diversity over a long period, a historical baseline is needed to validate the present data against. Historical otolith samples provide a unique source of DNA that can be used for analyzing temporal genetic stability. An important parameter in this context of conservation genetics is the effective population size, N_e, which determines the potential risk of losing genetic diversity (Nielsen & Hansen 2008).

The sole (*Solea solea)* provides an excellent model for studying population connectivity and demographic stability in the North-East Atlantic Ocean. One particular region has been the target of heavy beam trawl fishery since the 1950s, namely the North Sea. Hence, historical time series of fisheries data and archived otolith collections are readily available for this region and may provide insights into the stability of the populations in this region. Nevertheless, little knowledge is available on the population structure, connectivity between spawning grounds and nursery grounds, and (effective) population size. Therefore, the aim of this thesis was to provide knowledge on the population connectivity and demographic stability of sole.

In the next section, the major findings of all previous chapters are summarized and it is shown how a multi-disciplinary approach as applied here can improve the knowledge on sole populations under high fishing pressure. Additionally several management implications are compiled and a number of suggestions for future research formulated to safeguard the evolutionary resilience of sole.

CONNECTIVITY

In the first part of this thesis we investigated the contemporary connectivity of *Solea solea* populations in the North-East Atlantic using complementary approaches at various spatio-temporal scales. Initially, we performed a broad scale population genetic analysis using a set of ten microsatellite markers and part of the mitochondrial cytochrome *b* gene (chapter 1). Otolith microchemistry and otolith shape were subsequently applied to investigate the connectivity and traceability (chapters 2-4). In chapter 5 the results from the genetic markers were combined with the otolith microchemistry results to evaluate the power of the combined approach. Based on the results of part I, summarized in Fig. 1 and Fig. 2, I propose the following theses (T1-T4) about sole migration and connectivity.

- **T1: Sole juveniles show only limited movement after larval settlement.**

Juvenile sole from four different nursery areas in the Southern Bight of the North Sea were characterized by a distinct otolith elemental composition, leading to an overall high assignment success of 88 % (chapter 2). Although various studies have analysed the microchemical composition of sole, they focused on a local scale, whereas almost nothing is known about the connectivity at the larger scale of the North Sea or between local populations across the Dover Strait. Our results indicated that the microchemical variation was higher between samples at opposite sides of the southern North Sea than between samples along the same coastline, although a high assignment success was obtained for all nursery grounds. The observed spatial differences in otolith microchemistry imply that there are environmental differences between locations and that juvenile sole stay long enough at the nursery ground in order to establish a typical fingerprint.

Our conclusion of limited juvenile dispersal is consistent with other studies. Low mobility of young sole after settlement has also been shown in the Thames estuary based on the

consistent relationships between stable isotope signals ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) of juvenile sole and their invertebrate prey and based on otolith microchemistry, in either estuarine or coastal habitats (Leakey et al. 2008; Leakey et al. 2009). Physical tagging of *Solea solea* in the North Sea and English Channel confirmed the limited movement of juveniles (Burt & Millner 2008). This was not only observed in the North Sea, but also in other areas within its geographical distribution range. Stable isotopes ($\delta^{13}C$, $\delta^{15}N$) pointed to distinct isotopic signatures between nursery areas and high nursery fidelity for 0-group juveniles, but an enhanced mobility after the first year of life in a Portuguese estuary (Vinagre et al. 2008). Otolith microchemistry confirmed the limited movement of juvenile sole along the Portuguese coast (Vasconcelos 2007). In the Bay of Biscay, otolith microchemistry assigned juveniles with high success to the Loire and Gironde estuary, demonstrating nursery fidelity (de Pontual et al. 2000).

The reason why the young flatfish remain concentrated in coastal and estuarine areas is most likely the high abundance of food and higher temperature, resulting in fast growth. This enables them to escape from predators (such as crustaceans, fish and birds), reduce the window that they are vulnerable to predation and enhance survival during winter (van der Veer et al. 2000; Amara 2004). In later stages, the increased locomotory capacity combined with higher energy demands lead to a larger foraging area (Vinagre et al. 2008).

- **T2: Juvenile sole at the nursery grounds in the North Sea are the progeny of a large homogenous spawning group.**

In theory, genetic differentiation among groups of juveniles might result from the 'sweepstakes reproductive success' (Hedgecock 1994). Under those circumstances, young recruits are the result from only a limited number of adults that were able to match their spawning behaviour to favourable oceanographic conditions. The successful parents may vary greatly among years, resulting in temporal variation which is often higher than the spatial variation. Genetic patchiness may also result from habitat dependent selective processes causing differential survival of certain genotypes (Guinand et al. 2008). Furthermore, if larval dispersal is limited, the genetic structure of juveniles would reflect the nearby adult spawning populations and show reduced genetic variation.

Based on the microsatellite markers used in our study, the juvenile sole from the different nurseries did not differ genetically (chapter 1). We further compared the genetic

composition of the juvenile sole with the adult sole and observed similar levels of genetic diversity. The absence of distinct genetic differentiation between juvenile samples from different nurseries might indicate either that adult fish form a single homogenous population or that juveniles arriving and settling at the nursery grounds are a mixture of progeny from distinct populations. Since no signal of strong genetic differentiation among adult samples could be detected within the North Sea, we believe that the first hypothesis is more likely. Probably the genetic differentiation observed between adult aggregations within the North Sea was also too low for an accurate assignment of juveniles to the adult populations (Hedgecock et al. 2007).

Absence of genetic differentiation among juvenile sole (0- and 1- group) was also observed in a study by Guinand et al. (2008) using three intronic markers and in a study on plaice (*Pleuronectes platessa*) using microsatellite markers (Hoarau et al. 2002). A simulation study, modelling the transport of sole larvae showed possible connections between some spawning sites and more distant nurseries, depending on the dominant hydrodynamics and vertical migration behaviour of the larvae (Savina et al. 2010). It is therefore not excluded that nurseries receive larvae from several spawning aggregations.

- **T3: There is local self-recruitment off the UK coast and higher connectivity off continental Europe.**

In chapter 4 otolith microchemistry was used to identify the nursery origin of the adult sole, in order to assess the relative contribution of several European nursery grounds and investigate the amount of self-recruitment. From this analysis, it seemed that a majority of adult sole sampled off the Thames were allocated to the nurseries off the UK coast, suggesting local recruitment and some site fidelity. Adults sampled in the German Bight showed a juvenile microchemical fingerprint that was most similar to the adjacent Wadden Sea nursery (Texel). These results are consistent with a tagging study of sole in the North Sea, showing a high degree of site fidelity; juvenile sole recruited mainly to the adult population of the same area (Burt & Millner 2008). The hypothesis of local recruitment is also supported by the recruitment variability observed among the different sole stocks (Rijnsdorp et al. 1992).

Against our expectations, most samples from the Belgian spawning grounds were assigned to English nursery grounds. Almost none of the samples showed the nursery signal of the Scheldt estuary, while this is thought to be an important nursery ground for young fish such as sole (Beyst et al. 1999). We believe that this is due to the unbalanced sampling design: we are missing juvenile samples from the Belgian Coast and from the inner German Bight in our analysis. Therefore, it is too early at this stage to draw any conclusions about the relative contribution of the different nursery grounds. To improve this study, more nursery grounds should be included, including the German Bight and the Belgian Coast. Furthermore, the sampling design should be expanded in time to prepare a 'library' of elemental fingerprints for each year class to show that temporal variation in elemental composition is absent or negligible.

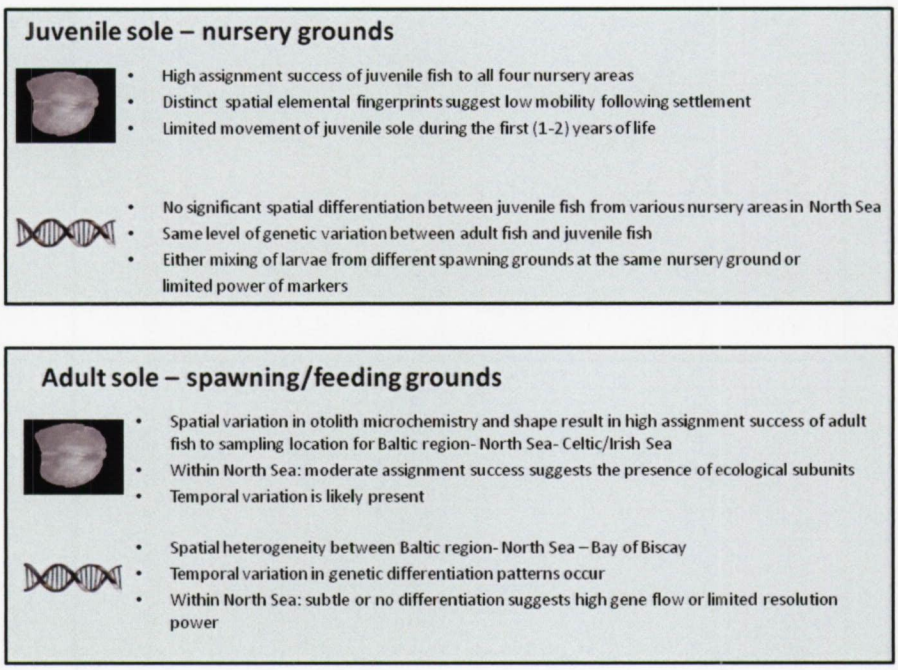

Fig. 1 *Solea solea.* Summary of the inferences from otolith markers and from molecular markers regarding juvenile sole (upper panel) and adult sole (lower panel).

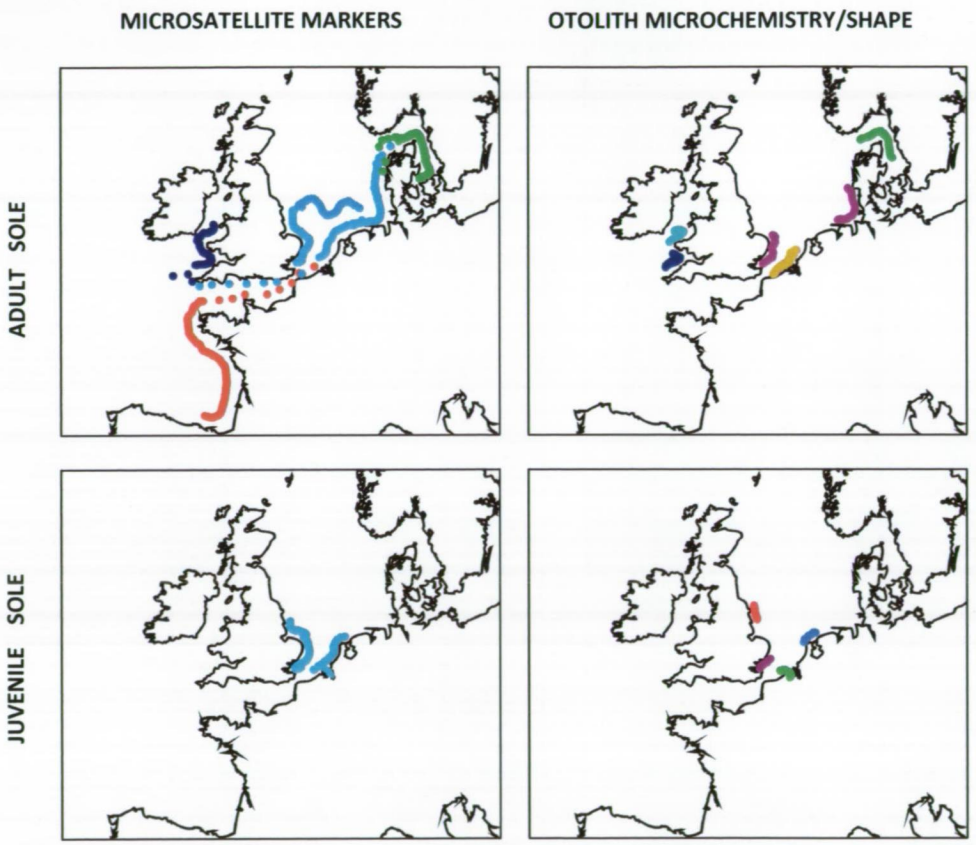

Fig. 2 *Solea solea.*
Summary of the results of microsatellite markers (left panels) and otolith microchemistry & shape (right panels). Identical colours indicate homogeneity; different colours indicate significant differences. Dotted lines indicate possible gene flow. For microchemistry/shape of adults, assignments are based on otolith edge with assignments higher than 70 % indicated with a different colour. Note the isolation-by-distance pattern with microsatellite markers (adults), with strong genetic differences between Bay of Biscay and Skagerrak/Kattegat, but smaller differences between North Sea and Irish Sea/Biscay. Juvenile sole are characterized by a homogeneous genotypic signal but distinct otolith microchemistry.

- **T4: There is population structure of adult sole at large spatial scale, with potential barriers to gene flow in the Skagerrak and the Bay of Biscay; and high connectivity within the North Sea.**

The strongest genetic divergence was obvious at the largest spatial scale, namely between the Bay of Biscay and the Kattegat/Skagerrak (chapter 1). This pattern was supported by the correlation between geographic and genetic distance based on microsatellite markers and predominantly determined by the samples from the most northern region. A barrier to gene flow seems to be present between the Skagerrak and the rest of the North Sea populations. Even though the differentiation between the Bay of Biscay and the North Sea was weaker, some indication of genetic heterogeneity among those regions was apparent. The sole sampled at different locations within the Southern Bight of the North Sea showed only subtle genetic differentiation, without temporal stability (chapter 1).

In general, our findings from the otolith microchemistry and shape analyses (chapter 3) supported the results from the genetic markers, namely large spatial differences between the regions 'Kattegat and Skagerrak' and the Southern Bight of the North Sea. Because there were no otolith samples from the Bay of Biscay, it is currently impossible to tell if otolith markers can also discriminate sole from the Bay of Biscay. Compared to genetic markers, otolith microchemistry had a higher assignment success within the North Sea (chapter 3 & 5). Otolith shape alone turned out to be less accurate in assigning fish to their sampling location. A summary of the inferences from both types of markers is presented in figure 1 and figure 2.

Large-scale spatial homogeneity throughout much of the North-East Atlantic with an isolation-by-distance pattern in some cases seems to be the general pattern observed in many marine fishes of the NE Atlantic. A similar pattern of isolation by geographic distance (IBD) was found in earlier population genetic studies of sole by Kotoulas et al. (1995) and Cabral et al. (2003) using allozyme markers. This is also detected in cod (*Gadus morhua*) (Hutchinson et al. 2001), flounder (*Platichthys flesus*) (Hemmer-Hansen et al. 2007b) and herring (*Clupea harengus*) (Mariani et al. 2005). The genetic structuring and limited gene flow observed in many species between North Sea and the transition to the Baltic could have a biological and evolutionary significance. The Skagerrak, Kattegat and the Belt Sea form the transition between the highly saline North Sea and the brackish waters of the Baltic Sea. Because of these specific environmental conditions, selection might play an important role

as an evolutionary force creating population differences in that area (Larsen et al. 2007; Nielsen et al. 2009b).

Also historical events following the last glaciations may have contributed to the observed genetic structure of sole. During the last glacial maximum (15-25 kya), an ice sheet covered the North Atlantic Ocean, North Sea and Baltic Sea, forcing species southwards into one of the refugial areas to avoid extinction. In the south, refuges have been suggested around the Iberian Peninsula (Gysels et al. 2004), Azores (Chevolot et al. 2006) and the Mediterranean Sea (Domingues et al. 2007). Smaller refugia have been suggested in the Bay of Biscay (Nesbø et al. 2000), the southwestern Irish coast, Hurd Deep in the English Channel and at the edges of the Baltic ice sheets (Koljonen et al. 1999). After the temperature increased, recolonization from these refugial areas took place. The connection between the Baltic and North Sea was established around 8-9 kya. The mitochondrial network analysis of *Solea solea* (chapter 1) showed a typical star-like pattern suggesting a recent population expansion. It seems that individuals coming from the North Sea have colonized the Baltic transition region about 8000 years ago and the high haplotype diversity was maintained thanks to the high N_e (see chapter 7) and absence of barriers.

The absence of strong differences between North Sea sole aggregations might reflect patterns of high gene flow. Alternatively to the hypothesis of gene flow, it could also be explained by the high effective population size, preventing strong effects of genetic drift. Not only other flatfishes such as plaice (*Pleuronectes platessa*) (Hoarau et al. 2004; Was et al. 2010), turbot (*Scophthalmus maximus*) (Nielsen et al. 2004) and flounder (*Platichthys flesus*) (Borsa et al. 1997; Hemmer-Hansen et al. 2007b) revealed this pattern of high gene flow and genetic homogeneity; also in whiting (*Merlangius merlangus*) (Charrier et al. 2007), sand goby (*Pomatoschistus minutus*) (Larmuseau et al. 2010b) and herring (*Clupea harengus*) (Larsson et al. 2007), similar patterns are observed based on microsatellite or allozyme markers.

- **Summary: Connectivity model for *Solea solea***

Populations can be linked by the exchange of larvae, juveniles, young recruits or adults. Based on our results using genetic markers, otolith microchemistry and otolith shape, we propose the following connectivity model for *Solea solea* between spawning and nursery grounds in the North Sea (Fig. 3):

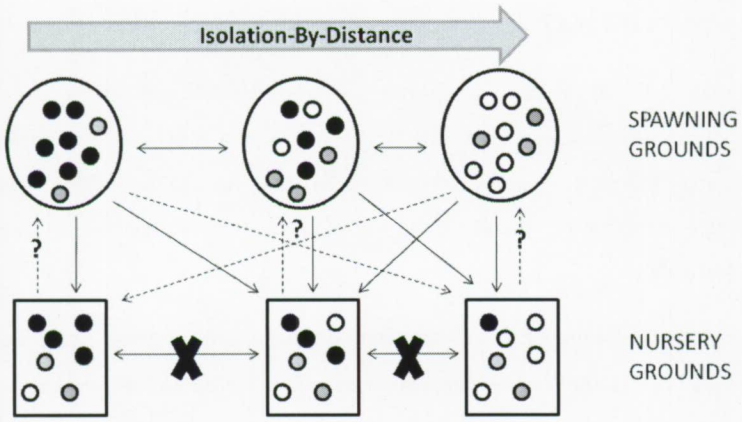

Fig. 3 Proposed migration model for *Solea solea* in the North Sea. Arrows indicate directions of movement. Dotted arrows indicate uncertainties. Coloured dots represent individuals with different genotypes. Adjacent spawning grounds are genetically more similar because of higher gene flow.

The spawning aggregations are not fully discrete, but follow an isolation-by-distance pattern across the North-East Atlantic. Within the North Sea, there is large genetic homogeneity due to high levels of gene flow or due to the high effective population size. About the fate of the larvae originating from the different spawning grounds, we can only speculate. Possibly, there is an exchange of genetic material through larval dispersal, depending on the local hydrodynamic conditions and the larval behavior. Larvae from the various spawning aggregations likely settle in the nearby nursery grounds, resulting in a mixed genetic composition of juvenile sole at the nursery grounds. Once settled, movement of the juvenile fish is restricted during their first years of life, which results in a distinct otolith microchemical profile because of environmental differences in water chemistry. It remains a question whether the (sub)adults from different nurseries recruit to a common feeding ground and later on return to the same spawning grounds year after year (homing). Our preliminary results based on otolith microchemistry suggest that subadults likely recruit to local spawning populations (Chapter 4). This is also suggested by physical tagging experiments (Rijnsdorp et al. 1992). Physical tagging studies have also shown that adult sole undertake relatively short movements away from their spawning grounds (75 km – 150 km) (Burt & Milner 2008), which leads us to suspect that most exchange among populations happens during larval and/or subadult stages.

PLENTY OF FISH IN THE SEA?

In 2007, about 28 % of the global monitored stocks were either overexploited, depleted or recovering from depletion, while more than half of the stocks were fully exploited, producing catches close to their sustainable limits (FAO 2008). The drastic reductions in population size of marine fish increases the risk of losing genetic variability through genetic drift (Boehlert 1996).

The joint analysis of phenotypic and genetic data from the same archived otoliths provides a way to study the evolutionary consequences of exploitation. Given the invaluable information that can be extracted from otoliths, it is crucial to develop reliable methods to jointly collect phenotypic and genetic information from an otolith without damaging it. In chapter 6, we optimized a protocol for the extraction of DNA from historical otoliths and showed that the choice of the extraction method may have significant effects on the success of using these otoliths for subsequent age and growth analyses.

In the North Sea, the shift from otter trawl to beam trawl in the early 1960s resulted in increased mortality rates for flatfish, in particular for sole (Rijnsdorp et al. 2008). Despite the decrease of spawning stock biomass with more than 50 % since the late 1950s (ICES 2009) and the indications for rapid phenotypic changes over 50 years of intense fishing (Mollet et al. 2007), we could not detect any long-term decrease in neutral genetic diversity (neither in allelic richness nor expected heterozygosity) in North Sea sole between 1957 and 2007. Furthermore, the effective population size was estimated for North Sea sole for the first time ever; our results suggested that N_e is most likely larger than 2000, implying that genetic drift does not have a major effect on neutral genetic diversity in this population (chapter 7). Does this mean that there are still plenty of fish in the sea and that there is no need for ringing any alarm bells for sole? To answer this, additional factors need to be taken in consideration.

First, although it is difficult to measure N_e in large populations because of the many methodological assumptions (such as absence of migration), it is mainly the ratio of N_e/N_c which has profound implications for conservation. A low ratio of effective size to census size suggests greater vulnerability to changes in genetic diversity, patterns of genetic differentiation and responses to environmental changes. Thus, even if the N_e estimate

obtained in this study for North Sea sole was higher than 2000, the N_e/N_c ratio remained very low (10^{-5}). This means that, despite a very high census size of the population (in the order of millions) much fewer individuals actually contribute to the next generation. Additionally, it cannot be predicted which individuals will represent the successful spawners. Successful spawners are often the older and larger fish (Wright & Trippel 2009) that also produce more offspring (Palumbi 2004a). In several flatfish the egg size and weight are proportional to female age and size with potential consequences for larval viability (Buckley et al. 1991; Rijnsdorp & Vingerhoed 1994; Witthames et al. 1995). Therefore, it is very important to maintain a broad age-structure (Hauser & Carvalho 2008) in order to preserve healthy fish stocks for the future.

Second, little is known about the relationship between neutral variation and adaptive variation. Neutral markers represent only one aspect of genetic variation and they are not necessarily linked to knowledge on adaptive genetic changes in response to anthropogenic and natural evolutionary drivers (Nielsen & Hansen 2008). The absence of any detectable loss in neutral variation is probably related to the large effective population size. If the population is as large as suggested here, it is likely affected by selection because in large populations, selection is relatively more important than genetic drift (Nielsen et al. 2009a). If the fishery is selectively removing the older and larger individuals, certain heritable traits (e.g. affecting growth and the age of reproduction) are likely to change due to fisheries-induced selection (Law 2007). Fishing can thus act negatively upon the sole stock through fisheries-induced selection, even without strongly reducing the effective population size.

To conclude, the significant discrepancy between the effective size and census size observed in sole indicates that monitoring genetic diversity requires more attention in fisheries management. It is clear that the potential loss of genetic diversity (either due to human-induced selection pressures or due to low effective population size) can have undesirable consequences both for the health of the fish populations but also from the human perspective, because it results in a loss of yield (Kenchington et al. 2003).

MANAGEMENT IMPLICATIONS

On the potential of traceability tools

Our study identified the combined use of otolith microchemistry and shape as the most powerful traceability tool for identifying sole over a large geographical range in the North-East Atlantic region (chapter 3). In general, our results pointed to three main groups: Kattegat/Skagerrak, North Sea and Celtic Sea/Irish Sea with distinct differences between groups and more subtle differences between populations within groups. These results were confirmed with neutral genetic markers (chapter 1 & chapter 5), although the discrimination power was lower, probably due to the large effective population size (chapter 7), the substantial level of gene flow or due to the limited number of loci genotyped.

The use of otolith microchemistry and shape to trace back the origin of landed fish (whole specimens) at a moderate spatial scale seems promising to assist fisheries enforcement initiatives. It might provide much needed complementary information on suspected fraud when genetics lacks small scale resolution. Yet, for the tracing of market fillets, headless fish or processed products, these markers are of course not applicable and genetic markers are more promising.

Before using a marker in a legal framework, it requires validation and extensive quality testing (Ogden 2008). It is absolutely necessary that results can be reproduced among laboratories. The gradual shift from microsatellite markers to SNP markers not only increases the assignment power of genetic markers but also enables a good validation (Ogden 2008). Shape morphometrics is also an easy and practical tool because it only requires a picture of the otolith (taken in a certain orientation and with a certain quality) but it does not require much standardization procedures. Otolith microchemistry on the other hand requires careful handling and a strict standardization between instruments (LA-ICPMS) and laboratories. An in depth validation is therefore necessary before this can be used in practice. Moreover, a marker is only valid as a traceability tool if temporal variation in the signal is negligible compared to spatial variation. This last point should be investigated more carefully for sole, since some temporal variation in the North Atlantic Ocean was detected (chapter 3).

Mismatch in fisheries management units for sole?

Current management units for sole are based on geo-economically defined ICES divisions (www.ices.dk); they distinguish among the North Sea (IV), the Skagerrak, Kattegat and the Belt Sea (IIIa, subdivision 22-24), Irish Sea (VIIa), Celtic Sea (VIIf and g), Gulf of Biscay (VIIIa,b). Sole in the Eastern English Channel (VIId) and the Western English Channel (VIIe) are also managed in a separate division. Further, there are also divisions in the Southwest and West of Ireland. These management units are associated with the collection areas for fisheries statistics and were originally not developed in relation to population biology or integrity.

In general, the incorporation of genetic data into fisheries models is rare. This is probably because genetic markers draw inferences about populations on an evolutionary time scale, while fisheries management is more interested in short-term demographic independence (Waples et al. 2008). Furthermore, before population genetics can become a useful tool in fisheries management, the patterns of genetic structure should be temporally stable. Decisions on management units should be based on a combination of genetic data (from different markers) and ecological/demographic data (Florin & Höglund 2008).

In our study, there was no evidence for strong mismatches between biological units and management units for sole based on the microsatellite markers (chapter 1). Such mismatches have been documented in other species in the NE Atlantic such as cod, herring, haddock, whiting, blue whiting and European hake (Reiss et al. 2009). There were no genetically distinct subpopulations of sole that were managed together. On the contrary, the number of management units seemed larger than the number of biological units. Based on the neutral genetic markers, there was only evidence for three or four management units: the Baltic region (Skagerrak, Kattegat and Belt Sea), the North Sea and English Channel, and the Bay of Biscay. Possibly, the Irish and Celtic Sea represent a separate management unit.

Given that not all our samples were spawning samples, we cannot exclude the possibility of the existence of subpopulations of sole in the North Sea. It seems that a substantial amount of gene flow among the populations within the North Sea prevents strong local genetic structure. Also the samples that we included from the Eastern (VIId) and Western (VIIe) English Channel were taken after the spawning season (July/August) and therefore we could

not make any strong inferences on the genetic heterogeneity of the spawning fish in these areas. Improvements to our study would involve the inclusion of spawning samples from these locations.

Marine Protected Areas (MPA)

Marine reserves have been proposed as an efficient management tool to protect and conserve biodiversity and encourage the sustainable use of resources (Halpern 2003; Palumbi 2004b). In the North-East Atlantic, OSPAR is developing a network of MPAs which should be established by 2010, complementing the protected areas for the marine environment established under the EU Habitats Directive and EU Birds Directive (OSPAR 2010).

If part of a marine system is closed for fishing, the fisheries outside the reserve might benefit from it through the emigration of adult or juvenile fish across the boundaries of the reserve (i.e. spillover effect) or through the export of eggs and larvae (Gell & Roberts 2003). It has been shown for a number of species that no-take MPAs can protect against fisheries-induced selection for early maturation (Baskett et al. 2005).

Although MPA's will have the most benefits for species that are relatively sedentary, several studies indicated that marine reserves could also be beneficial for more mobile species (Gell & Roberts 2003). If there is some level of site-fidelity and a proportion of the population remains within a relatively small area, these residents may build up biomass and reproductive capacity within the reserve, even if some individuals undertake longer migrations. Highly migratory species might benefit from reserves that target the places where they are extremely vulnerable, such as at the nursery grounds (Gell & Roberts 2003).

The limited juvenile movement and residence of *Solea solea* in local coastal nursery areas, highlights the importance of the protection and quality of such habitats. Habitat degradation and pollution of shallow coastal areas and estuaries might negatively affect sole recruitment. Some site fidelity of adult sole was also suggested through the use of otolith microchemistry. Furthermore, because the beam trawl fishery can have a significant negative impact on the benthic prey communities (Rabaut 2009, but see Hinz et al. 2008), the closure of certain areas to fishing might benefit the sole indirectly, through the positive effects on its prey.

The reserve size is dependent on the scales of movements of the species that need to be protected (Gell & Roberts 2003). Nevertheless, in order to provide benefits to mobile fish species such as sole, not only the size of the marine reserve is important, it is also necessary to use it in conjunction with other management measures such as catch and effort control (Stefansson & Rosenberg 2006) and the mandatory use of sorting devices on fishing gears to prevent the catches of undersized fish.

Climate change

Possible effects of climate change on sole were not within the scope of this study. However, it is known that the maintenance of large genetic diversity is important to react or adapt to changing environments (Frankham et al. 2002). Furthermore, a good knowledge on current population dynamics and connectivity is important to predict effects of environmental changes. For instance, the genetic and ecological heterogeneity observed between the Skagerrak/Kattegat sole and the North Sea sole suggests little connectivity among these regions. If sole would disappear from one region, a substantial amount of genetic diversity would be lost and recolonization from other areas might be difficult.

Like in many marine fish, the physical environment plays a fundamental role in the life of sole. Especially events during early life (e.g. the time of spawning, the duration of the egg and larval stage, survival of larvae etc.) depend on a range of abiotic factors such as water temperature, salinity and hydrodynamics. Temperature is probably one of the most important factors, influencing growth and mortality. The range in temperature tolerance of sole eggs has been experimentally determined from 8°C to 16°C (Irvin 1974). Because sole in the Southern North Sea reaches the northern limit of its geographic distribution, it might benefit from an increase in water temperature (Henderson & Seaby 2005). Increasing winter temperatures in the southeastern North Sea significantly increased the growing period of sole and in combination with higher temperatures during the growing period, this results in higher growth rate (Teal et al. 2008). A time series (1980-2003) in the Bristol Channel pointed to a positive correlation between the abundance of young sole in autumn and sea water temperature during the hatching season. Juvenile growth in the Bristol Channel and the Thames is also positively correlated with the North Atlantic Oscillation Index for the winter months prior to spawning (NAOWI), possibly linked through an increased food abundance due to increased productivity in years with a positive NAO index (Attrill & Power

2002; Henderson & Seaby 2005). On the other hand, in the North Sea strong year classes of sole occurred after a strong winter, although extreme winters can induce high mortality (Rijnsdorp et al. 1992). North Sea sole exhibits a negative relationship between temperature and recruitment (Cook & Heath 2005). It seems that sole from different regions might respond differently to temperature. Changes in sea water temperature might also affect adult sole differently, for instance through a shift in the timing of spawning.
Coinciding with the climate predictions of less severe winters and a gradual rise in sea surface temperature, other species such as the solenette (*Buglossidium luteum*) might expand northwards, thereby affecting the distribution and abundance of *Solea solea* through predation and competition (van Hal et al. 2010).

To summarize, climate change will possibly exert an effect on sole through changes in the timing of spawning, shifts in distribution range, alterations of the foodweb and the community, and changes in ocean currents.

PERSPECTIVES

The present study has contributed to the general knowledge on the population connectivity and genetic stability in *Solea solea,* but our results also highlight the need and the opportunities for further research. We will therefore conclude with some recommendations for future research.

Temporal stability of otolith derived data

Understanding the temporal stability of the otolith microchemical signal is essential to reconstruct individual migration histories. In marine fish, interannual differences in otolith microchemistry have been observed, potentially confounding spatial differences. In some cases temporal variability was high, but in other cases spatial trends were comparable among cohorts (Gillanders 2002b; Patterson et al. 2008; Schaffler & Winkelman 2008). Chapter 2 and 3 describe some temporal variation in sole otolith microchemistry. Future assignment and full connectivity studies should therefore take into account the temporal aspects by increasing the number of temporal replicates.

Include stable isotope markers

Besides trace elements, stable isotope ratio analysis in otoliths can be used to reconstruct migration histories of fish (Panfili et al. 2002, Gao & Beamish 2003). The inclusion of stable isotope markers in otoliths of sole might permit a more accurate assignment of individuals to their capture location. Commonly used isotopic markers are $\delta^{18}O$ and $\delta^{13}C$ (Thorrold et al. 2001, Rooker et al. 2008, Schloesser et al. 2010). Most of the variation in oxygen isotope ratios is due to temperature differences in the water. Otolith $\delta^{13}C$ values are linked to metabolic rates and can thus be used to discriminate fish with different food sources or to study ontogenetic changes (Ashford & Jones 2007, Hidalgo et al. 2008).

Local adaptation and fisheries-induced selection

Our data on neutral genetic markers will serve as a baseline for future studies examining the effects of selection using non-neutral genetic markers. Although neutral microsatellite markers suggest genetic homogeneity, significant heterogeneity might still exist in adaptive variation (Hauser & Carvalho 2008). One of the primary future objectives should be to search for evidence of local adaptation in *Solea solea*. Local adaptation are 'the processes leading to fitness advantages of local genotypes in comparison with non-resident genotypes' (Nielsen et al. 2009a). Adaptive differences arise due to the effect of selection and in large populations such differences might occur much faster than differences due to genetic drift (Conover et al. 2006). Selective drivers might be of natural or anthropogenic origin. A classic approach for demonstrating local adaptation includes common garden experiments. Another strategy is to study variation in candidate genes. Suitable candidate genes may include genes linked to growth, maturity or immunity (e.g. MHC) (Nielsen et al. 2009a). Local adaptation has been observed for a number of marine fish species with similar levels of neutral genetic differentiation, such as the sand goby (Larmuseau et al. 2009; Larmuseau et al. 2010a), European flounder (Hemmer-Hansen et al. 2007a; Larsen et al. 2007), herring (André et al. 2010) and cod (Nielsen et al. 2009b).

The archived DNA collection can be further analyzed to study adaptive changes through time. Such changes in adaptive variation can be correlated with fishing intensity and selectivity to investigate fisheries-induced evolution (Nielsen et al. 2009a). It is suggested that the changes towards an earlier maturation at a smaller size observed in several marine fish are due to the selective nature of fishing (Kuparinen & Merilä 2007; Law 2007).

Comparison of genetic N_e with demographic N_e

Our estimate of effective population size based on genetic markers should be validated with estimates obtained from demographic data. Demographic N_e estimates can be calculated using demographic parameters such as fluctuating population size, sex ratio and variance in family size (Ardren & Kapuscinski 2003; Walsh & Lynch in press). This should help to identify the factors responsible for the low N_e/N_c ratio in sole.

Larger coverage of the genome

Recent advances in technology have produced a shift from microsatellite markers towards SNP markers (Single Nucleotide Polymorphism). SNPs are sites in the genome with single base changes in the DNA sequence. Although individual SNP markers are less informative than microsatellites, the analysis of a high number of SNP markers generates higher quality data and a better coverage of the genome because SNPs occur at a higher density in the genome and have lower genotyping error rates (Vignal et al. 2002). The identification of variation at individual nucleotide sites will likely improve the power and resolution in relation to population structure and traceability of sole (Stokstad 2010).

Coupling of genetic models to hydrodynamic models

Modelling the oceanographic forces that control larval dispersal can help to explain the observed genetic population structure (White et al. 2010). Simulations of larval trajectories should be used to estimate probabilities of larval dispersal and to explain ultimately the observed genetic structure. The challenges of hydrodynamic modelling will be the validation with field observations which require extensive egg or larval surveys. Recent studies combining genetic models with hydrodynamic models show the potential of this approach in understanding connectivity patterns (polychaetes (Jolly et al. 2009) and molluscs (Gilg & Hilbish 2003; White et al. 2010), corals (Galindo et al. 2006), crustaceans (Galindo et al. 2010), fish (Selkoe et al. 2010)).

Comprehensive phylogeographical study

Finally, mitochondrial sequencing provides an opportunity to investigate in greater detail the historical demography and population history of Atlantic sole. Combining our data with sequences from the Mediterranean Sea would certainly present a good opportunity to study recolonization patterns of *Solea solea* in relation to the Last Glacial Maximum.

ADDENDUM: OVERVIEW OF SAMPLES

Overview of samp les: Sample code, location, area, year and month of sampling, adult (A) or juvenile (J), geographic position of sampling location, number of samples, with indication of chapters wherein samples are used.

Sample Code	Full name location	Area/Sea	Year	Month	A/J	Latitude	Longitude	N	CH1	CH2	CH3	CH4	CH5
STO07	Great Belt (Store Belt)	Belt Sea	2007	Oct	A	55°10'29"N	11°02'44"E	48	x				
KATA07	Kattegat	Kattegat	2007	Nov	A	57°08'91"N	11°38'52"E	48	x		x		x
KATB07	Kattegat	Kattegat	2007	Nov	A	56°25'31"N	12°11'21"E	48	x				
SKA07	Skagerrak	Skagerrak	2007	Nov	A	58°09'43"N	9°30'32"E	48	x		x		x
GER07	German Bight	North Sea	2007	May	A	54°31'12"N	7°53'23"E	60	x		x	x	x
LINC08	Lincolnshire coast	North Sea	2008	Aug	A	53°19'96"N	0°25'63"E	52	x				
NOR07	Norfolk banks	North Sea	2007	Aug	A	53°00'70"N	1°33'62"E	46	x				
NOR08	Norfolk banks	North Sea	2008	Aug	A	53°00'70"N	1°33'62"E	28	x				
THA07	off Thames	North Sea	2007	Aug	A	51°27'80"N	1°20'00"E	96	x		x	x	x
THA08	off Thames	North Sea	2008	Aug	A	51°27'80"N	1°20'00"E	63	x				
BEL07S	Belgian coast	North Sea	2007	May	A	51°23'22"N	3°10'01"E	96	x		x	x	x
BEL07F	Belgian coast	North Sea	2007	Aug	A	51°21'14"N	2°55'45"E	80	x				
BEL08S	Belgian coast	North Sea	2008	May	A	51°23'22"N	3°10'01"E	96	x		x	x	
BEL08F	Belgian coast	North Sea	2008	Aug	A	51°21'14"N	2°55'45"E	60	x				
CEL08	Celtic Sea	Celtic Sea	2008	Apr	A	50°49'00"N	5°01'00"W	96	x		x		x
IS08	Irish Sea	Irish Sea	2008	Mar	A	52°13'00"N	5°20'00"W	96	x		x		x
ENG08	Eastern English Channel	English Channel	2008	Jul	A	50°46'54"N	1°29'04"E	58	x				
WCH09	Western English Channel	English Channel	2009	Aug	A	49°39'41"N	2°07'38"W	80	x				
BISA07	Bay of Biscay	Atlantic Ocean	2007	Mar	A	46°53'00"N	2°47'00"W	95	x				
BISB07	Bay of Biscay	Atlantic Ocean	2007	Mar	A	45°36'00"N	1°24'00"W	61	x				
BISC07	Bay of Biscay	Atlantic Ocean	2007	Mar	A	46°20'00"N	1°53'00"W	48	x				
LINC07	Lincolnshire coast	North Sea	2007	Aug	J	53°19'96"N	0°25'63"E	96	x	x		x	
TEX06	Texel	Wadden Sea	2006	Aug	J	52°58'11"N	4°56'35"E	58	x	x		x	
TEX07	Texel	Wadden Sea	2007	May	J	52°58'11"N	4°56'35"E	48	x				
THA07J	off Thames	North Sea	2007	Aug	J	51°27'80"N	1°20'00"E	35	x	x		x	
ZAN06	Zandvliet	Scheldt estuary	2006	Sep	J	51°23'50"N	4°06'59"E	74	x	x		x	
ZAN07	Zandvliet	Scheldt estuary	2007	Oct	J	51°23'50"N	4°06'59"E	61	x	x		x	
BEL08J	Belgian coast	North Sea	2008	May	J	51°23'22"N	3°10'01"E	36	x				
Total								**1811**	**1811**	**152**	**244**	**277**	**535**

SUMMARY

Connectivity can be defined as the extent to which populations are linked by the exchange of larvae, juveniles or adults across a species' range. It plays a fundamental role in local population dynamics and the genetic structure of a species. A good understanding of connectivity is important for population resiliency against changing environments and exploitation, the design of marine reserves and the sustainable conservation of a species.

The sole (***Solea solea***), a common marine flatfish inhabiting the North-East Atlantic, is currently under high fishing pressure. It has a long history of exploitation in the North Sea. Since the early 1960s, mortality rates increased considerably due to the introduction of the beam trawl, which increased the catch efficiency for flatfish. Because sole has always been a target species of the North Sea beam trawl fishery, long time series of fisheries data and large collections of historical otoliths (earstones) are available in fisheries institutes. These otoliths were initially collected for ageing purposes but the adhering dried tissue also represents a unique source of historical DNA.

Despite the commercial importance of sole, little is known about its population structure and connectivity in the North-East Atlantic Ocean. Furthermore, it was not clear whether the intensification of the fishery has also led to changes in the genetic diversity and if the evolutionary potential of the species is being threatened.
This study investigated the **connectivity and demographic genetic stability** of sole in the North-East Atlantic Ocean. In the first part of the thesis, genetic markers and ecological markers were used to assess the population structure, while the second part examined the genetic stability of sole in the North Sea under high fishing pressure.

A thorough population genetic analysis using neutral microsatellite markers and a mitochondrial marker showed **genetic differences** at a **large scale**, along a latitudinal gradient from the Skagerrak/Kattegat to the Bay of Biscay. At a smaller spatial scale within the North Sea, the subpopulations seemed genetically homogeneous, probably due to a high level of gene flow and/or the high effective population size preventing strong effects of genetic drift (Chapter 1). Besides genetic markers, **otolith microchemistry** may be applied as a tool for studying population connectivity because it reflects environmental differences experienced by a fish. The analysis of the elemental composition of juvenile sole otoliths showed that fish living in different nurseries had a specific elemental composition.

We concluded that the **movement of juvenile sole**, once settled in a nursery ground, is rather **limited** (Chapter 2). Subsequently otolith microchemistry and otolith shape were used to discriminate between adult sole from different spawning grounds. Otolith microchemistry was a successful marker for tracing fish back to their spawning site but especially the **combination of otolith microchemistry with otolith shape provided a succesfull traceability tool** (Chapter 3). The integration of genetic markers and otolith microchemistry further improved the assignment for some populations (Chapter 5). The movement of individuals from **juvenile to adult habitats** provides another critical link. Chapter 4 explored the assignment of adults to their source nursery based on the microchemistry of the juvenile portion of the otolith. This is important to assess the relative contribution of various nursery grounds. A relatively high percentage of self-recruitment was found, suggesting that young fish recruit to the adjacent adult stocks.

In the **second part** of the thesis the temporal genetic stability of the North Sea sole was analyzed using a collection of historical otoliths (dating back to the 1950s). Because of their high value and uniqueness, it is important to obtain as much information as possible (phenotypic and genetic) from these otoliths. Therefore, a DNA extraction protocol was first optimized in Chapter 6. Subsequently, we examined whether a decrease in genetic diversity has taken place due to the intensification of the fishery since the 1960s. A **remarkable genetic stability** was found from the 1950s up to present. This was reflected in the **high effective population size** estimate (N_e > 2000) obtained for the North Sea sole, using different methods. The N_e represents an important parameter in conservation genetics because it determines the amount of genetic diversity that can be lost due to genetic drift. In theory, the estimate means that genetic drift is probably not an important evolutionary driver in this population. Nevertheless, the ratio of N_e/N_c (census size) was very small and comparable to other marine fish. It is typical for organisms with a type III survival pattern, indicating that drift may occur in populations with high census counts. We conclude that the maintenance of a large population size of mixed age classes (including older individuals) provides the best guarantee for the conservation of sole stocks in the North-East Atlantic Ocean.

SAMENVATTING

Connectiviteit geeft aan in welke mate populaties in de verschillende delen van het leefgebied van een soort verbonden zijn door de uitwisseling van larven, juvenielen of adulten. Het speelt een fundamentele rol in de lokale populatiedynamieken en genetische structuur van een soort. Een goede kennis van connectiviteit is dan ook van belang voor het inschatten van de weerbaarheid van populaties tegen milieuschommelingen en exploitatie, voor het afbakenen van mariene reservaten en voor het duurzaam beheer van een soort.

De **tong (*Solea solea*)** is een mariene platvissoort die momenteel intens bevist wordt. Deze soort kent een lange exploitatiegeschiedenis in de Noordzee. In het begin van de jaren 1960 vond er een sterke mortaliteitstoename plaats ten gevolge van de introductie van de boomkor. Dit vistuig zorgde voor een forse toename van de vangstefficiëntie van platvis. Omdat tong steeds een belangrijke doelsoort is geweest van de boomkorvisserij in de Noordzee, bestaan er voor dit gebied lange tijdsreeksen van visserijgegevens en zijn er historische collecties van otolieten (gehoorsteentjes) beschikbaar in visserij-instituten. Deze otolieten werden voornamelijk verzameld ten behoeve van leeftijdsbepalingen, maar recent is ontdekt dat ze tevens een unieke bron van DNA vormen, wegens het nog aanwezige restweefsel.

Hoewel tong een belangrijke commerciële soort uitmaakt voor de boomkorvisserij, is er weinig geweten over de populatiestructuur en connectiviteit in de Noordoost Atlantische Oceaan. Bovendien is niet gekend of de intense visserijdruk gezorgd heeft voor veranderingen in de genetische diversiteit, met name of het evolutionair potentieel van deze soort gevaar loopt.

Deze studie onderzocht daarom de **connectiviteit en de demografische genetische stabiliteit** van tong in de Noordoost Atlantische Oceaan. In het eerste luik van de thesis (Hoofdstuk 1 t.e.m. 5) werden signalen omtrent de populatiestructuur opgespoord met behulp van genetische en ecologische merkers. In het tweede luik werd onderzocht of de genetische diversiteit van tong inderdaad beïnvloed werd door de hoge visserijdruk (Hoofdstuk 6 en 7).

Aan de hand van een grondige analyse van microsatellietmerkers en een mitochondriale merker werd een **significante genetische differentiatie** gevonden voor tong en dit over een grote regionale schaal langsheen een latitudinale gradiënt van Skagerrak en Kattegat tot aan de Golf van Biskaje. Op kleinere schaal, binnen de Noordzee, waren de populaties genetisch homogener, wat wellicht te verklaren valt door een substantiële genmigratie tussen subpopulaties en/of door de hoge effectieve populatiegrootte waardoor de effecten van genetische drift beperkt blijven (Hoofdstuk 1). Naast genetische merkers, kan eveneens de **microchemische samenstelling van otolieten** aangewend worden als merker voor het onderscheiden van aparte visstocks, omdat elk van hen het signaal weerspiegelt van omgevingsverschillen in de concentratie van spoorelementen. Het analyseren van de elementsamenstelling van juveniele tongotolieten toonde aan dat dit geochemische signaal zeer specifiek was voor een bepaald juveniel habitat. Dit wijst er sterk op dat de **migratie van jonge tong**, eens de kinderkamer opgezocht, zeer waarschijnlijk **beperkt** is (Hoofdstuk 2). Vervolgens werd nagegaan of otoliet microchemie en otolietvorm ook gebruikt kunnen worden om de herkomst van volwassen tong, in functie van de verschillende paaigronden, te onderscheiden. Otoliet microchemie bleek in vele gevallen een succesvolle merker voor het traceren van vissen, maar vooral de **combinatie van microchemie en otolietvorm bleek een succesvolle 'tracer'** te zijn (Hoofdstuk 3). Het **integreren** van zowel genetische merkers als otoliet merkers leverde in sommige gevallen nog een verbetering op van het toewijzen van adulte tong aan hun vangstplaats (Hoofdstuk 5). Een volgend belangrijk aspect is de **relatie tussen adulte paaiaggregaties en juveniele kinderkamers**. In hoofdstuk 4 werd nagegaan of we op basis van otolietmicrochemie van de juveniele regio in de otoliet konden vaststellen van welke kinderkamer een volwassen tong afkomstig is. Dit is belangrijk indien we de relatieve bijdrage van verschillende kinderkamers willen evalueren. De resultaten toonden een relatief hoog percentage van zelf-rekrutering, wat vermoedelijk betekent dat jonge vissen aansluiten bij een lokale stock van volwassen dieren.

In het **tweede luik** onderzochten we de temporele genetische stabiliteit van tong in de Noordzee. Er werd gebruik gemaakt van een historische verzameling otolieten vanaf 1950. Omdat deze historische stalen zeer waardevol en uniek zijn, is het belangrijk dat zoveel mogelijk fenotypische en genetische informatie bekomen wordt. Hiertoe werd eerst een DNA opzuiveringsmethode geoptimaliseerd, die geen schade toebrengt in functie van

verdere fenotypische analyses (Hoofdstuk 6). Tenslotte werd nagegaan of een daling in genetische diversiteit heeft plaatsgevonden ten gevolge van het intensifiëren van de visserij op tong sedert 1960. Er werd een **opmerkelijk genetische stabiliteit** waargenomen tijdens de periode 1950 tot op heden. Dergelijke stabiliteit weerspiegelde zich eveneens in de **grote effectieve populatiegrootte** (N_e > 2000) voor Noordzee tong, berekend aan de hand van verschillende methodes. De N_e vormt een belangrijke parameter voor het natuurbehoud omdat deze bepaalt in welke mate genetische diversiteit verloren kan gaan ten gevolge van genetische drift. In principe betekent deze berekende hoge N_e dat genetische drift in de tongpopulatie nog geen belangrijke evolutiefactor is. Toch bleek de verhouding van effectieve populatiegrootte ten opzichte van census grootte zeer klein en vergelijkbaar met die van andere mariene vissen. Dit is zeer typisch voor organismen met een type III overlevingscurve en het impliceert dat genetische drift zelfs kan optreden in populaties met hoge censusgrootte. Naar het visserijbeheer toe is het aangewezen om een ruime populatie in stand te houden opgebouwd uit verschillende leeftijdsklassen (inclusief oudere dieren). Dit levert immers een optimale garantie voor genetisch gezonde en duurzame tongpopulaties.

REFERENCES

Able KW (2005) A re-examination of fish estuarine dependence: Evidence for connectivity between estuarine and ocean habitats. Estuarine Coastal and Shelf Science, 64, 5-17.

Adcock GJ, Ramirez JHB, Hauser L, Smith P, Carvalho GR (2000) Screening of DNA polymorphisms in samples of archived scales from New Zealand snapper. Journal of Fish Biology, 56, 1283-1287.

Aguirre H, Lombarte A (1999) Ecomorphological comparisons of sagittae in *Mullus barbatus* and *M. surmuletus*. Journal of Fish Biology, 55, 105-114.

Allendorf FW, England PR, Luikart G, Ritchie PA, Ryman N (2008) Genetic effects of harvest on wild animal populations. Trends in Ecology & Evolution, 23, 327-337.

Amara R (2004) 0-Group flatfish growth conditions on a nursery ground (Bay of Canche, Eastern English Channel). Hydrobiologia, 518, 23-32.

Amara R, Lagardere F, Desaunay Y, Marchand JR (2000) Metamorphosis and estuarine colonisation in the common sole, *Solea solea* (L.): implications for recruitment regulation. Oceanologica Acta, 23, 469-484.

Andersson L (1996) Trends in nutrients and oxygen concentrations in the Skagerrak-Kattegat. Journal of Sea Research, 35, 63-71.

André C, Larsson LC, Laikre L, Bekkevold D, Brigham J, Carvalho GR, Dahlgren TG, Hutchinson WF, Mariani S, Mudde K, Ruzzante DE, Ryman N (2010) Detecting population structure in a high gene-flow species, Atlantic herring (*Clupea harengus*): direct, simultaneous evaluation of neutral vs putatively selected loci. Heredity, Advance online publication.

Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G (2008) LOSITAN: A workbench to detect molecular adaptation based on a F_{ST}-outlier method. BMC Bioinformatics, 9, 323.

Araki H, Waples RS, Ardren WR, Cooper B, Blouin MS (2007) Effective population size of steelhead trout: influence of variance in reproductive success, hatchery programs, and genetic compensation between life-history forms. Molecular Ecology, 16, 953-966.

Ardren WR, Kapuscinski AR (2003) Demographic and genetic estimates of effective population size (Ne) reveals genetic compensation in steelhead trout. Molecular Ecology, 12, 35-49.

Arkhipkin AI, Schuchert PC, Danyushevsky L (2009) Otolith chemistry reveals fine population structure and close affinity to the Pacific and Atlantic oceanic spawning grounds in the migratory southern blue whiting (*Micromesistius australis australis*). Fisheries Research, 96, 188-194.

Arslan Z, Secor DH (2005) Analysis of trace transition elements and heavy metals in fish otoliths as tracers of habitat use by American eels in the Hudson river estuary. Estuaries, 28, 382-393.

Ashford JR, Arkhipkin AI, Jones CM (2006) Can the chemistry of otolith nuclei determine population structure of Patagonian Toothfish *Dissostichus Eleginoides*? Journal of Fish Biology, 69, 708-721.

Ashford J, Jones C (2007) Oxygen and carbon stable isotopes in otoliths record spatial isolation of Patagonian toothfish (*Dissostichus eleginoides*). Geochimica et Cosmochimica Acta, 71, 87-94.

Ashford JR, Jones CM, Hofmann E, Everson I, Moreno C, Duhamel G, Williams R (2005) Can otolith elemental signatures record the capture site of Patagonian toothfish (*Dissostichus Eleginoides*), a fully marine fish in the Southern Ocean? Canadian Journal of Fisheries and Aquatic Sciences, 62, 2832-2840.

Attrill MJ, Thomes RM (1995) Heavy metal concentrations in sediment from the Thames Estuary, UK. Marine Pollution Bulletin, 30, 742-744.

Attrill MJ, Power M (2002) Climatic influence on a marine fish assemblage. Nature, 417, 275-278.

Bacevičius E, Karalius S (2008) A common sole *(Solea solea* Linnaeus, 1758): Actinopterygii: Pleuronectiformes: Soleidae) caught in the coastal zone of Lithuania. Acta Zoologica Lituanica, 18, 174-179.

Baden SP, Neil DM (2003) Manganese accumulation by the antennule of the Norway lobster *Nephrops norvegicus* (L.) as a biomarker of hypoxic events. Marine Environmental Research, 55, 59-71.

Baeyens W (1998) Evolution of trace metal concentrations in the Scheldt estuary (1978-1995). A comparison with estuarine and ocean levels. Hydrobiologia, 366, 157-167.

Baeyens W, Gillain G, Decadt G, Elskens I (1987) Trace metals in the eastern part of the North Sea. 1. Analyses and short-term distributions. Oceanologica Acta, 10, 169-179.

Baeyens W, Leermakers M, De Gieter M, Nguyen HL, Parmentier K, Panutrakul S, Elskens M (2005) Overview of trace metal contamination in the Scheldt estuary and effect of regulatory measures. Hydrobiologia, 540, 141-154.

Banks MA, Eichert W (2000) WHICHRUN (version 3.2): A computer program for population assignment of individuals based on multilocus genotype data. Journal of Heredity, 91, 87-89.

References

Barot S, Heino M, O'Brien L, Dieckmann U (2004) Estimating reaction norms for age and size at maturation when age at first reproduction is unknown. Evolutionary Ecology Research, 6, 659-678.

Baskett ML, Levin SA, Gaines SD, Dushoff J (2005) Marine reserve design and the evolution of size at maturation in harvested fish. Ecological Applications, 882-901.

Beaumont MA, Nichols RA (1996) Evaluating loci for use in the genetic analysis of population structure. Proceedings of the Royal Society of London Series B-Biological Sciences, 263, 1619-1626.

Beck MW, Heck KL, Able KW, Childers DL, Eggleston DB, Gillanders BM, Halpern B, Hays CG, Hoshino K, Minello TJ, Orth RJ, Sheridan PF, Weinstein MR (2001) The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. Bioscience, 51, 633-641.

Bedford BC (1983) A method for preparing sections of large numbers of otoliths embedded in black polyester resin. Journal Du Conseil, 41, 4-12.

Beebee T, Rowe G (2008) An introduction to molecular ecology, Second edn. Oxford University Press, New York. 400 pp.

Bekkevold D, Andre C, Dahlgren TG, Clausen LAW, Torenstensen E (2005) Environmental correlates of population differentiation in Atlantic herring. Evolution, 59, 2656-2668.

Belkhir K, Borsa P, Goudet J, Chickhi L, Bonhomme F (2004) Genetix v. 4.01, Logiciel sous WindowsTM pour la génétique des populations Laboratoire Génome et Population, Université Montpellier 2, Montpellier.

Berkeley SA, Chapman C, Sogard SM (2004) Maternal age as a determinant of larval growth and survival in a marine fish, *Sebastes melanops*. Ecology, 85, 1258-1264.

Beyst B, Mees J, Cattrijsse A (1999) Early postlarval fish in the hyperbenthos of the Dutch Delta (south-west Netherlands). Journal of the Marine Biological Association of the United Kingdom, 79, 709-724.

Boe R, Rise L, Ottesen D (1998) Elongate depressions on the southern slope of the Norwegian Trench (Skagerrak): morphology and evolution. Marine Geology, 146, 191-203.

Boehlert GW (1996) Biodiversity and the sustainability of marine fisheries. Oceanography, 9, 28-35.

Borremans C (1985) North Sea spawning grounds of the sole (*Solea solea*) located from the 1984 Belgian plankton survey.

Borsa P, Blanquer A, Berrebi P (1997) Genetic structure of the flounders *Platichthys flesus* and *P. stellatus* at different geographic scales. Marine Biology, 129, 233-246.

Braber L, de Groot SJ (1973). The food of five flatfish species (Pleuronectiformes) in the southern North Sea. Netherlands Journal of Sea Research, 6, 163-172.

Bradbury IR, Campana SE, Bentzen P (2008) Estimating contemporary early life-history dispersal in an estuarine fish: integrating molecular and otolith elemental approaches. Molecular Ecology, 17, 1438-1450.

Bradbury IR, Snelgrove PVR, Pepin P (2003) Passive and active behavioural contributions to patchiness and spatial pattern during the early life history of marine fishes. Marine Ecology-Progress Series, 257, 233-245.

Brophy D, Jeffries TE, Danilowicz BS (2004) Elevated manganese concentrations at the cores of clupeid otoliths: possible environmental, physiological, or structural origins. Marine Biology, 144, 779-786.

Brown JA (2006) Using the chemical composition of otoliths to evaluate the nursery role of estuaries for English sole *Pleuronectes vetulus* populations. Marine Ecology-Progress Series, 306, 269-281.

Bruford MW, Hanotte O, Brookfield JFY, Burke T (1992) Single locus and multilocus DNA fingerprinting. In: Molecular Genetic Analysis of Populations- A Molecular Approach (Hoelzel AE ed), pp. 227-229. Oxford: IRL Press.

Buckley LJ, Smigielski AS, Halavik TA, Caldarone EM, Burns BR, Laurence GC (1991) Winter Flounder *Pseudopleuronectes americanus* reproductive success. 2. Effects of Spawning time and female size on size, composition and viability of eggs and larvae. Marine Ecology-Progress Series, 74, 125-135.

Buonaccorsi VP, Mcdowell JR, Graves JE (2001) Reconciling patterns of inter-ocean molecular variance from four classes of molecular markers in blue marlin (*Makaira nigricans*). Molecular Ecology, 10, 1179-1196.

Burt GJ, Millner RS (2008) Movement of sole in the southern North Sea and eastern English Channel from tagging studies (1955-2004). Science Series Technical Report, Cefas Lowestoft, 144, 44 pp.

Branch TA, Watson R, Fulton EA, Jennings S, McGilliard CR, Pablico GT, Ricard D, Tracey SR (2010). The trophic fingerprint of marine fisheries. Nature, 468, 431-435.

Briggs J (online first) Marine extinctions and conservation. Marine Biology, 1-4.

Cabral HN, Castro F, Linhares D, Alexandrino P (2003) Genetic differentiation of *Solea Solea* (Linnaeus, 1758) and *Solea Senegalensis* Kaup, 1858, (Pisces : Pleuronectiformes) from several estuarine systems of the Portuguese coast. Scientia Marina, 67, 43-52.

Caley MJ, Carr MH, Hixon MA, Hughes TP, Jones GP, Menge BA (1996) Recruitment and the local dynamics of open marine populations. Annual Review of Ecology and Systematics, 27, 477-500.

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Marine Ecology-Progress Series, 188, 263-297.

Campana SE, Casselman JM (1993) Stock discrimination using otolith shape analysis. Canadian Journal of Fisheries and Aquatic Sciences, 50, 1062-1083.

Campana SE, Chouinard GA, Hanson JM, Frechet A, Brattey J (2000) Otolith elemental fingerprints as biological tracers of fish stocks. Fisheries Research, 46, 343-357.

Campana SE, Fowler AJ, Jones CM (1994) Otolith elemental fingerprinting for stock identification of Atlantic Cod (*Gadus Morhua*) using Laser-Ablation ICPMS. Canadian Journal of Fisheries and Aquatic Sciences, 51, 1942-1950.

Campana SE, Neilson JD (1985) Microstructure of fish otoliths. Canadian Journal of Fisheries and Aquatic Sciences, 42, 1014-1032.

Campana SE, Thorrold SR (2001) Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? Canadian Journal of Fisheries and Aquatic Sciences, 58, 30-38.

Cardinale M, Doering-Arjes P, Kastowsky M, Mosegaard H (2004) Effects of sex, stock, and environment on the shape of known-age Atlantic cod (*Gadus morhua*) otoliths. Canadian Journal of Fisheries & Aquatic Sciences, 61, 158-167.

Chambers RC, Leggett WC (1996) Maternal influences on variation in egg sizes in temperate marine fishes. American Zoologist, 36, 180-196.

Champalbert G, Koutsikopoulos C (1995) Behavior, transport and recruitment of Bay of Biscay sole (*Solea solea*) - laboratory and field studies. Journal of the Marine Biological Association of the United Kingdom, 75, 93-108.

Chao A, Shen T-J (2010) Program SPADE (Species Prediction And Diversity Estimation). Program and User's Guide published at http://chao.stat.nthu.edu.tw.

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. Molecular Biology and Evolution, 24, 621-631.

Charrier G, Coombs SH, McQuinn IH, Laroche J (2007) Genetic structure of whiting *Merlangius merlangus* in the northeast Atlantic and adjacent waters. Marine Ecology-Progress Series, 330, 201-211.

Charters RA, Lester RJG, Buckworth RC, Newman SJ, Ovenden JR, Broderick D, Kravchuk O, Ballagh A, Welch DJ (2010) The stock structure of grey mackerel *Scomberomorus semifasciatus* in Australia as inferred from its parasite fauna. Fisheries Research, 101, 94-99.

Chevolot M, Ellis JR, Rijnsdorp AD, Stam WT, Olsen JL (2008) Temporal changes in allele frequencies but stable genetic diversity over the past 40 years in the Irish Sea population of thornback ray, *Raja clavata*. Heredity, 101, 120-126.

Chevolot M, Hoarau G, Rijnsdorp AD, Stam WT, Olsen JL (2006) Phylogeography and population structure of thornback rays (*Raja clavata* L., Rajidae). Molecular Ecology, 15, 3693-3705.

Christensen JM (1964) Burning of otoliths, a technique for age determination of soles and other fish. Journal du Conseil, 29: 73-81.

Clark J (1968) Seasonal movements of striped bass contingents of Long Island sound and the New York bight. Transactions of the American Fisheries Society, 97, 320-343.

Clarke KR (1993) Nonparametric multivariate analyses of changes in community structure. Australian Journal of Ecology, 18, 117-143.

Conover DO, Arnott SA, Walsh MR, Munch SB (2005) Darwinian fishery science: lessons from the Atlantic silverside (*Menidia menidia*). Canadian Journal of Fisheries and Aquatic Sciences, 62, 730-737.

Conover DO, Clarke LM, Munch SB, Wagner GN (2006) Spatial and temporal scales of adaptive divergence in marine fishes and the implications for conservation. Journal of Fish Biology, 69, 21-47.

Cook RM, Heath MR (2005) The implications of warming climate for the management of North Sea demersal fisheries. ICES Journal of Marine Science, 62, 1322-1326.

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity of marine populations: Open or closed? Science, 287, 857-859.

Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. Annual Review of Marine Science, 1, 443-466.

Crawford NG (2010) SMOGD: software for the measurement of genetic diversity. Molecular Ecology Resources, 10, 556-557.

Danielssen DS, Edler L, Fonselius S, Hernroth L, Ostrowski M, Svendsen E, Talpsepp L (1997) Oceanographic variability in the Skagerrak and Northern Kattegat, May-June, 1990. ICES Journal of Marine Science, 54, 753-773.

De Clerck R, Van de Velde J (1973) A study of the spawning and nursery areas of soles along the Belgian coast. ICES report of Demersal Fish Committee.

de Pontual H, Lagardere F, Amara R, Bohn M, Ogor A (2003) Influence of ontogenetic and environmental changes in the otolith microchemistry of juvenile sole (*Solea solea*). Journal of Sea Research, 50, 199-210.

de Pontual H, Lagardere F, Troadec H, Batel A, Desaunay Y, Koutsikopoulos C (2000) Otoliths imprinting of sole (*Solea Solea)* from the Bay of Biscay: a tool to discriminate individuals from nursery origins? Oceanologica Acta, 23, 497-513.

Dellwig O, Bosselmann K, Kölsch S, Hentscher M, Hinrichs J, Böttcher ME, Reuter R, Brumsack HJ (2007) Sources and fate of manganese in a tidal basin of the German Wadden Sea. Journal of Sea Research, 57, 1-18.

Dempster AP, Laird NM, Rubin DB (1977) Maximum likelihood from incomplete data via the EM algorithm. Journal of the Royal Statistical Society Series B, 39, 1-38.

Depestele J, Polet H, Van Craeynest K, Vandendriessche S (2009) An overview of sea trials with the alternative beam trawl. ILVO Research Report, Oostende, Belgium.

de Roos AM, Boukal DS, Persson L (2006) Evolutionary regime shifts in age and size at maturation of exploited fish stocks. Proceedings of the Royal Society B-Biological Sciences, 273, 1873-1880.

DeWoody JA, Avise JC (2000) Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. Journal of Fish Biology, 56, 461-473.

Dieckmann U, Heino M (2007) Probabilistic maturation reaction norms: their history, strengths, and limitations. Marine Ecology-Progress Series, 335, 253-269.

Domingues VS, Santos RS, Brito A, Alexandrou M, Almada VC (2007) Mitochondrial and nuclear markers reveal isolation by distance and effects of Pleistocene glaciations in the northeastern Atlantic and Mediterranean populations of the white seabream *(Diplodus sargus*, L.). Journal of Experimental Marine Biology and Ecology, 346, 102-113.

Dunlop ES, Enberg K, Jorgensen C, Heino M (2009) Toward Darwinian fisheries management. Evolutionary Applications, 2, 246-259.

Durieux EDH, Begout ML, Pinet P, Sasal P (2010) Digenean metacercariae parasites as natural tags of habitat use by 0-group common sole *Solea solea* in nearshore coastal areas: A case study in the embayed system of the Pertuis Charentais (Bay of Biscay, France). Journal of Sea Research, 64, 107-117.

Eastwood PD, Meaden GJ, Grioche A (2001) Modelling spatial variations in spawning habitat suitability for the Sole *Solea solea* using regression quantiles and GIS procedures. Marine Ecology-Progress Series, 224, 251-266.

Eastwood PD, Meaden GJ, Carpentier A, Rogers SI (2003) Estimating limits to the spatial extent and suitability of sole (*Solea solea*) nursery grounds in the Dover Strait. Journal of Sea Research, 50, 151-165.

El Mousadik A, Petit RJ (1996) High level of genetic differentiation for allelic richness among populations of the argan tree *Argania spinosa* (L. Skeels) endemic to Morocco. Theoretical and Applied Genetics, 92, 832-836.

Elsdon TS, Gillanders BM (2003) Reconstructing migratory patterns of fish based on environmental influences on otolith chemistry. Reviews in Fish Biology and Fisheries, 13, 219-235.

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: Hypotheses, assumptions, limitations and inferences. Oceanography and Marine Biology: An Annual Review, 46, 297-330.

Engelhard GH (2008) One hundred and twenty years of change in fishing power of English North Sea trawlers. In: Advances in fisheries science, pp. 1-25. Blackwell Publishing, Oxford.

Estoup A, Largiader CR, Perrot E, Chourrout D (1996) Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. Molecular Marine Biology and Biotechnology, 5, 295-298.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology, 14, 2611-2620.

Exadactylos A, Geffen AJ, Panagiotaki P, Thorpe JP (2003) Population structure of Dover sole *Solea solea*: RAPD and allozyme data indicate divergence in European stocks. Marine Ecology-Progress Series, 246, 253-264.

Exadactylos A, Geffen AJ, Thorpe JP (1998) Population structure of the Dover sole, *Solea solea* L., in a background of high gene flow. Journal of Sea Research, 40, 117-129.

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evolutionary Bioinformatics, 1, 47-50.

FAO (2008) The state of the world fisheries and aquaculture (ed. FAO Fisheries and Aquaculture Department). Food and Agriculture Organisation of the United Nation, Rome, 2009.

Feyrer F, Hobbs J, Baerwald M, Sommer T, Yin QZ, Clark K, May B, Bennett W (2007) Otolith microchemistry provides information complementary to microsatellite DNA for a migratory fish. Transactions of the American Fisheries Society, 136, 469-476.

Florin AB, Höglund J (2007) Absence of population structure of turbot (*Psetta maxima*) in the Baltic Sea. Molecular Ecology, 16, 115-126.

Florin AB, Höglund J (2008) Population structure of flounder (*Platichthys flesus*) in the Baltic Sea: differences among demersal and pelagic spawners. Heredity, 101, 27-38.

Fogarty MJ, Botsford LW (2007) Population connectivity and spatial management of marine fisheries. Oceanography, 20, 112-123.

Fonds M (1979) Laboratory observations on the influence of temperature and salinity on development of the eggs and growth of the larvae of *Solea solea* (Pisces). Marine Ecology-Progress Series, 1, 91-99.

Fontes J, Caselle JE, Sheehy MS, Santos RS, Warner RR (2009) Natal signatures of juvenile *Coris julis* in the Azores: investigating connectivity scenarios in an oceanic archipelago. Marine Ecology-Progress Series, 387, 51-59.

Förlin L, Baden SP, Eriksson S, Granmo Å, Lindesjöö E, Magnusson K, Ekelund R, Esselin A, Sturve J (1996) Effects of contaminants in roundnose grenadier (*Coryphaenoides rupestris*) and Norway lobster (*Nephrops norvegicus*) and contaminant levels in mussels (*Mytilus edulis*) in the Skagerrak and Kattegat compared to the Faroe Islands. Journal of Sea Research, 35, 209-222.

Forrester GE, Swearer SE (2002) Trace elements in otoliths indicate the use of open-coast versus bay nursery habitats by juvenile California halibut. Marine Ecology-Progress Series, 241, 201-213.

Francois O, Durand E (2010) Spatially explicit Bayesian clustering models in population genetics. Molecular Ecology Resources, 10, 773-784.

Frank KT, Brickman D (2001) Contemporary management issues confronting fisheries science. Journal of Sea Research, 45, 173-187.

Frankham R (1995a) Conservation genetics. Annual Review of Genetics, 29, 305-327.

Frankham R (1995b) Effective population size, adult population size ratios in wildlife- a review. Genetical Research, 66, 95-107.

Frankham R, Ballou JD, Briscoe DA (2002) Introduction to conservation genetics. Cambridge University Press, Cambridge, UK, 640 pp.

Franklin IR (1980) Evolutionary change in small populations. In: Conservation Biology, an evolutionary-ecological perspective (eds. Soule ME, Wilcox BA). Sinauer, Sunderland, MA, 135-149.

Franklin IR, Frankham R (1998) How large must populations be to retain evolutionary potential. Animal Conservation, 1, 69-73.

Fraser DJ, Hansen MM, Ostergaard S, Tessier N, Legault M, Bernatchez L (2007) Comparative estimation of effective population sizes and temporal gene flow in two contrasting population systems. Molecular Ecology, 16, 3866-3889.

Fromentin JM, Ernande B, Fablet R, de Pontual H (2009) Importance and future of individual markers for the ecosystem approach to fisheries. Aquatic Living Resources, 22, 395-408.

Funes V, Zuasti E, Catanese G, Infante C, Manchado M (2004) Isolation and characterization of ten microsatellite loci for Senegal sole (*Solea senegalensis* Kaup). Molecular Ecology Notes, 4, 339-341.

Gaines SD, Gaylord B, Gerber LR, Hastings A, Kinlan BP (2007) Connecting places: The ecological consequences of dispersal in the sea. Oceanography, 20, 90-99.

Galindo HM, Olson DB, Palumbi SR (2006) Seascape genetics: A coupled oceanographic-genetic model predicts population structure of Caribbean corals. Current Biology, 16, 1622-1626.

Galindo HM, Pfeiffer-Herbert AS, McManus MA, Chao Y, Chai F, Palumbi SR (2010) Seascape genetics along a steep cline: using genetic patterns to test predictions of marine larval dispersal. Molecular Ecology, 19, 3692-3707.

Gao YW, Beamish RJ (2003) Stable isotopic composition of otoliths from tagged Pacific halibut, *Hippoglossus stenolepis*. Environmental Biology of Fishes, 67, 253-261.

Garoia F, Guarniero I, Grifoni D, Marzola S, Tinti F (2007) Comparative analysis of AFLPs and SSRs efficiency in resolving population genetic structure of Mediterranean *Solea vulgaris*. Molecular Ecology, 16, 1377-1387.

Garoia F, Marzola S, Guarniero I, Trentini M, Tinti F (2006) Isolation of polymorphic DNA microsatellites in the common sole *Solea vulgaris*. Molecular Ecology Notes, 6, 144-146.

Gawarkiewicz G, Monismith S, Largier J (2007) Observing larval transport processes affecting population connectivity progress and challenges. Oceanography, 20, 40-53.

Geffen AJ, Jarvis K, Thorpe JP, Leah RT, Nash RDM (2003) Spatial differences in the trace element concentrations of Irish Sea plaice *Pleuronectes platessa* and whiting *Merlangius merlangus* otoliths. Journal of Sea Research, 50, 245-254.

Gell FR, Roberts CM (2003) Benefits beyond boundaries: the fishery effects of marine reserves. Trends in Ecology & Evolution, 18, 448-455.

Gilg MR, Hilbish TJ (2003) The geography of marine larval dispersal: Coupling genetics with fine-scale physical oceanography. Ecology, 84, 2989-2998.

Gillanders BM (2002a) Connectivity between juvenile and adult fish populations: do adults remain near their recruitment estuaries? Marine Ecology Progress Series, 240, 215-223.

Gillanders BM (2002b) Temporal and spatial variability in elemental composition of otoliths: implications for determining stock identity and connectivity of populations. Canadian Journal of Fisheries and Aquatic Sciences, 59, 669-679.

Gillanders BM, Kingsford MJ (2000) Elemental fingerprints of otoliths of fish may distinguish estuarine 'nursery' habitats. Marine Ecology-Progress Series, 201, 273-286.

Gonzalez-Salas C, Lenfant P (2007) Interannual variability and intra-annual stability of the otolith shape in European anchovy *Engraulis encrasicolus* (L.) in the Bay of Biscay. Journal of Fish Biology, 70, 35-49.

Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-statistics. Journal of Heredity, 86, 485-486.

Goudet J (2002) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available at http://www.unil.ch/izea/softwares/fstat.html. Updated from Goudet (1995).

Goudet J (2004) PCAGEN [computer program]. Available from http://wwww2.unil.ch/ popgen/softwares/ pcagen.html

Gower JC (1975) Generalized Procrustes Analysis. Psychometrika, 40, 33-51.

Grift RE, Rijnsdorp AD, Barot S, Heino M, Dieckmann U (2003) Fisheries-induced trends in reaction norms for maturation in North Sea plaice. Marine Ecology-Progress Series, 257, 247-257.

Grioche A, Koubbi P, Harlay X, Sautour B (2001) Sole larval distribution (*Solea solea*) in the eastern English Channel and Southern Bight of the North Sea. Journal of the Marine Biological Association of the United Kingdom, 81, 673-678.

Guarniero I, Franzellitti S, Ungaro N, Tommasini S, Piccinetti C, Tinti F (2002) Control region haplotype variation in the Central Mediterranean common sole indicates geographical isolation and population structuring in Italian stocks. Journal of Fish Biology, 60, 1459-1474.

Guinand B, Rolland JL, Bonhomme F (2008) Genetic structure of the common sole (*Solea solea*) in the Bay of Biscay: Nurseries as units of selection? Estuarine, Coastal and Shelf Science, 78, 316-326.

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics, 48, 361-372.

Gysels ES, Hellemans B, Patarnello T, Volckaert FAM (2004) Current and historic gene flow of the sand goby *Pomatoschistus minutus* on the European Continental Shelf and in the Mediterranean Sea. Biological Journal of the Linnean Society, 83, 561-576.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleid Acids Symposium Series, 41, 95-98.

Halpern BS (2003) The impact of marine reserves: Do reserves work and does reserve size matter? Ecological Applications, 13, S117-S137.

Hamer PA, Jenkins GP, Coutin P (2006) Barium variation in *Pagrus auratus* (Sparidae) otoliths: A potential indicator of migration between an embayment and ocean waters in south-eastern Australia. Estuarine Coastal and Shelf Science, 68, 686-702.

Hamerlynck O, Hostens K, Arellano RV, Mees J, Van Damme PA (1993) The mobile epibenthic fauna of soft bottoms in the Dutch delta (south-west Netherlands): Spatial structure. The Netherlands Journal of Aquatic Ecology, 27, 343-358.

Hansen MM, Bekkevold D, Jensen LF, Mensberg KLD, Nielsen EE (2006) Genetic restoration of a stocked brown trout *Salmo trutta* population using microsatellite DNA analysis of historical and contemporary samples. Journal of Applied Ecology, 43, 669-679.

Hansen MM, Kenchington E, Nielsen EE (2001) Assigning individual fish to populations using microsatellite DNA markers. Fish and Fisheries, 2, 93-112.

Harden Jones FR (1968) Fish migration St. Martin Press, New York, 325 pp.

Hardy OJ, Vekemans X (2002) SPAGEDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Molecular Ecology Notes, 2, 618-620.

Hartl DL, Clark AG (2007) Principles of population genetics (4th Edn). Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, USA, 545 pp.

Hauser L, Adcock GJ, Smith PJ, Ramirez JHB, Carvalho GR (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus)*. Proceedings of the National Academy of Sciences of the United States of America, 99, 11742-11747.

Hauser L, Carvalho GR (2008) Paradigm shifts in marine fisheries genetics: ugly hypotheses slain by beautiful facts. Fish and Fisheries, 9, 333-362.

Hauser L, Seamons TR, Dauer M, Naish KA, Quinn TP (2006) An empirical verification of population assignment methods by marking and parentage data: hatchery and wild steelhead (*Oncorhynchus mykiss*) in Forks Creek, Washington, USA. Molecular Ecology, 15, 3157-3173.

Heath D, Lou DC, Ovenden J, Begg GA (2007) Effect of DNA extraction on ageing success in coral trout (*Plectropomus leopardus*) otoliths. Journal of Fish Biology, 71, 302-307.

Hedgecock D (1994) Does variance in reproductive success limit effective population sizes of marine organisms. Genetics and Evolution of Aquatic Organisms, 122-134.

Hedgecock D, Barber PH, Edmands S (2007) Genetic approaches to measuring connectivity. Oceanography, 20, 70-79.

Hedrick P (2005) Large variance in reproductive success and the Ne/N ratio. Evolution, 59, 1596-1599.

Heino M (1998) Management of evolving fish stocks. Canadian Journal of Fisheries and Aquatic Sciences, 55, 1971-1982.

Hellberg ME, Burton RS, Neigel JE, Palumbi SR (2002) Genetic assessment of connectivity among marine populations. Bulletin of Marine Science, 70, 273-290.

Helsel DR, Hirsch RM (2002) Methods for data below the reporting limit. In: Statistical methods in water resources, Book 4, chapter A3, pp. 357-376. U.S. Geological Survey, Elsevier, Amsterdam.

Hemmer-Hansen J, Nielsen EE, Frydenberg J, Loeschcke V (2007a) Adaptive divergence in a high gene flow environment: Hsc70 variation in the European flounder (*Platichthys flesus* L.). Heredity, 99, 592-600.

Hemmer-Hansen J, Nielsen EE, Gronkjaer P, Loeschcke V (2007b) Evolutionary mechanisms shaping the genetic population structure of marine fishes; lessons from the European flounder (*Platichthys flesus* L.). Molecular Ecology, 16, 3104-3118.

Henderson PA, Seaby RM (2005) The role of climate in determining the temporal variation in abundance, recruitment and growth of Sole *Solea solea* in the Bristol Channel. Journal of the Marine Biological Association of the United Kingdom, 85, 197-204.

Hidalgo M, Tomas J, Hoie H, Morales-Nin B, Ninnemann US (2008) Environmental influences on the recruitment process inferred from otolith stable isotopes in *Merluccius merluccius* off the Balearic Islands. Aquatic Biology, 3, 195-207.

Higashitani T, Takatsu T, Nakaya M, Joh M, Takahashi T (2007) Maternal effects and larval survival of marbled sole *Pseudopleuronectes yokohamae*. Journal of Sea Research, 58, 78-89.

Higgins RM, Danilowicz BS, Balbuena JA, Danielsdottir AK, Geffen AJ, Meijer WG, Modin J, Montero FE, Pampoulie C, Perdiguero-Alonso D, Schreiber A, Stefansson MO, Wilson B (2010) Multi-disciplinary fingerprints reveal the harvest location of cod *Gadus morhua* in the northeast Atlantic. Marine Ecology-Progress Series, 404, 197-206.

Hill WG (1979) Note on effective population-size with overlapping generations. Genetics, 92, 317-322.

Hinz H, Hiddink JG, Forde J, Kaiser MJ (2008) Large-scale responses of nematode communities to chronic otter-trawl disturbance. Canadian Journal of Fisheries and Aquatic Sciences, 65, 723-732.

Hjort J (1914) Fluctuation in the great fisheries of northern Europe, viewed in the light of biological research. Rapport et Proces-Verbaux des Reunions du conseil permanent international pour l'exploration de la mer, 20, 1–228.

Hoarau G, Boon E, Jongma DN, Ferber S, Palsson J, Van Der Veer HW, Rijnsdorp AD, Stam WT, Olsen JL (2005) Low effective population size and evidence for inbreeding in an overexploited flatfish, plaice (*Pleuronectes platessa* L.). Proceedings of the Royal Society B-Biological Sciences, 272, 497-503.

Hoarau G, Piquet AMT, Van Der Veer HW, Rijnsdorp AD, Stam W, Olsen JL (2004) Population structure of plaice (*Pleuronectes platessa* L.) in northern Europe: a comparison of resolving power between microsatellites and mitochondrial DNA data. Journal of Sea Research, 51, 183-190.

References

Hoarau G, Rijnsdorp AD, Van der Veer HW, Stam WT, Olsen JL (2002) Population structure of plaice (*Pleuronectes platessa* L.) in northern Europe: microsatellites revealed large-scale spatial and temporal homogeneity. Molecular Ecology, 11, 1165-1176.

Hoelzel AR, Shivji MS, Magnussen J, Francis MP (2006) Low worldwide genetic diversity in the basking shark (*Cetorhinus maximus*). Biology Letters, 2, 639-642.

Holderegger R, Kamm U, Gugerli F (2006) Adaptive vs. neutral genetic diversity: implications for landscape genetics. Landscape Ecology, 21, 797-807.

Horwood J (2001) Population biology and ecology of the sole. Natural Resource Modeling, 14, 233-256.

Horwood J, Blaxter JHS, Southward AJ (1993) The Bristol Channel sole (*Solea solea* (L.)): A fisheries case study. Advances in Marine Biology, 29, 215-367.

Hostens K (2000) Spatial patterns and seasonality in the epibenthic communities of the Westerschelde (Southern Bight of the North Sea). Journal of the Marine Biological Association of the United Kingdom, 80, 27-36.

Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources, 9, 1322-1332.

Hutchings JA (2000) Collapse and recovery of marine fishes. Nature, 406, 882-885.

Hutchings JA, Baum JK (2005) Measuring marine fish biodiversity: temporal changes in abundance, life history and demography. Philosophical Transactions of the Royal Society B-Biological Sciences, 360, 315-338.

Hutchings JA, Reynolds JD (2004) Marine fish population collapses: consequences for recovery and extinction risk. Bioscience, 54, 297-309.

Hutchinson WF, Carvalho GR, Rogers SI (1999) A nondestructive technique for the recovery of DNA from dried fish otoliths for subsequent molecular genetic analysis. Molecular Ecology, 8, 893-894.

Hutchinson WF, Carvalho GR, Rogers SI (2001) Marked genetic structuring in localised spawning populations of cod *Gadus morhua* in the North Sea and adjoining waters, as revealed by microsatellites. Marine Ecology-Progress Series, 223, 251-260.

Hutchinson WF, van Oosterhout C, Rogers SI, Carvalho GR (2003) Temporal analysis of archived samples indicates marked genetic changes in declining North Sea cod (*Gadus morhua*). Proceedings of the Royal Society of London Series B-Biological Sciences, 270, 2125-2132.

ICES (2005a) Factors affecting the distribution of North Sea fish. ICES FishMap (www.ices.dk).

ICES (2005b) Report of the Working Group on the Application of Genetics in Fisheries and Mariculture (WGAGFM), 3-6 June 2005, Silkeborg, Denmark. ICES CM 2005/F:01. 47 pp.

ICES (2007) Report of the ICES Advisory Committee on Fishery Management, Advisory Committee on the Marine Environment and Advisory Committee on Ecosystems (2007). ICES Advice, Book 6, 249 pp.

ICES (2009) Report of the working group on the assessment of demersal stocks in the North Sea and Skagerrak - Combined spring and autumn (WGNSSK). ICES CM 2009/ACOM: 10, 1040.

ICES (2010) ICES Advice. Sole in subarea IV (North Sea).

Irvin DN (1974) Temperature tolerance of early developmental stages of Dover Sole, *Solea solea* (L.). In: Early life history of fish. Springer Berlin.

Iwata H, Ukai Y (2002) SHAPE: A computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. Journal of Heredity, 93, 384-385.

Iyengar A, Piyapattanakorn S, Stone DM, Heipel DA, Howell BR, Baynes SM, Maclean N (2000) Identification of microsatellite repeats in turbot (*Scophthalmus maximus*) and Dover sole (*Solea solea*) using a RAPD-based technique: characterization of microsatellite markers in Dover sole. Marine Biotechnology, 2, 49-56.

Jackson DA (1995) Protest- a Procrustean randomization test of community environment concordance. Ecoscience, 2, 292-303.

Jakobsdottir KB, Jorundsdottir OD, Skirnisdottir S, Hjorleifsdottir S, Hreggvidson GO, Danielsdottir AK, Pampoulie C. (2006) Nine new polymorphic microsatellite loci for the amplification of archived otolith DNA of Atlantic cod, *Gadus morhua* L. Molecular Ecology Notes, 6, 337-339.

Jarne P, Lagoda PJL (1996) Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution, 11, 424-429.

Jennings S, Kaiser MJ, Reynolds JD (2001) Marine fisheries ecology. Blackwell Science, 432 pp.

Johannesson K, Andre C (2006) Life on the margin: genetic isolation and diversity loss in a peripheral marine ecosystem, the Baltic Sea. Molecular Ecology, 15, 2013-2029.

Jolly MT, Guyard P, Ellien C, Gentil F, Viard F, Thiebaut E, Jollivet D (2009) Population genetics and hydrodynamic modeling of larval dispersal dissociate contemporary patterns of connectivity from

historical expansion into European shelf seas in the polychaete *Pectinaria koreni*. Limnology and Oceanography, 54, 2089-2106.

Jones GP, Milicich MJ, Emslie MJ, Lunow C (1999) Self-recruitment in a coral reef fish population. Nature, 402, 802-804.

Jones GP, Planes S, Thorrold SR (2005) Coral reef fish larvae settle close to home. Current Biology, 15, 1314-1318.

Jones GP, Srinivasan M, Almany GR (2007) Population connectivity and conservation of marine biodiversity. Oceanography, 20, 100-111.

Jonsdottir IG, Campana SE, Marteinsdottir G (2006a) Otolith shape and temporal stability of spawning groups of Icelandic cod (*Gadus morhua* L.). ICES Journal of Marine Science, 63, 1501-1512.

Jonsdottir IG, Campana SE, Marteinsdottir G (2006b) Stock structure of Icelandic cod *Gadus morhua* L. based on otolith chemistry. Journal of Fish Biology, 69, 136-150.

Jorde PE, Ryman N (2007) Unbiased estimator for genetic drift and effective population size. Genetics, 177, 927-935.

Jørgensen C, Enberg K, Dunlop ES, Arlinghaus R, Boukal DS, Brander K, Ernande B, Gardmark A, Johnston F, Matsumura S, Pardoe H, Raab K, Silva A, Vainikka A, Dieckmann U, Heino M, Rijnsdorp AD (2007) Ecology - Managing evolving fish stocks. Science, 318, 1247-1248.

Jørgensen HBH, Hansen MM, Bekkevold D, Ruzzante DE, Loeschcke V (2005) Marine landscapes and population genetic structure of herring (*Clupea harengus* L.) in the Baltic Sea. Molecular Ecology, 14, 3219-3234.

Jost L (2008) G_{ST} and its relatives do not measure differentiation. Molecular Ecology, 17, 4015-4026.

Kenchington E (2003) The effects of fishing on species and genetic diversity. In: Responsible Fisheries in the Marine Ecosystem (eds. Sinclair M, Valdimarsson G), p. 235-253. Food and Agriculture Organization of the United Nations/CABI Publishing, Rome.

Kenchington E, Heino M, Nielsen EE (2003) Managing marine genetic diversity: Time for action? ICES Journal of Marine Science, 60, 1172-1176.

Knutsen H, Andre C, Jorde PE, Skogen MD, Thuroczy E, Stenseth NC (2004) Transport of North Sea cod larvae into the Skagerrak coastal populations. Proceedings of the Royal Society of London Series B-Biological Sciences, 271, 1337-1344.

Knutsen H, Jorde PE, Andre C, Stenseth NC (2003) Fine-scaled geographical population structuring in a highly mobile marine species: the Atlantic cod. Molecular Ecology, 12, 385-394.

Koljonen ML, Jansson H, Paaver T, Vasin O, Koskiniemi J (1999) Phylogeographic lineages and differentiation pattern of Atlantic salmon (*Salmo salar*) in the Baltic Sea with management implications. Canadian Journal of Fisheries and Aquatic Sciences, 56, 1766-1780.

Koljonen ML, Pella JJ, Masuda M (2005) Classical individual assignments versus mixture modeling to estimate stock proportions in Atlantic salmon (*Salmo salar)* catches from DNA microsatellite data. Canadian Journal of Fisheries and Aquatic Sciences, 62, 2143-2158.

Koskinen MT, Sundell P, Piironen J, Primmer CR (2002). Genetic assessment of spatiotemporal evolutionary relationships and stocking effects in grayling (*Thymallus thymallus*, Salmonidae). Ecology Letters, 5, 193-205.

Kotoulas G, Bonhomme F, Borsa P (1995) Genetic structure of the common sole *Solea vulgaris* at different geographic scales. Marine Biology, 122, 361-375.

Koubbi P, Loots C, Cotonnec G, Harlay X, Grioche A, Vaz S, Martin C, Walkey M, Carpentier A (2006) Spatial patterns and GIS habitat modelling of *Solea solea*, *Pleuronectes flesus* and *Limanda limanda* fish larvae in the Eastern English Channel during the spring. Scientia Marina, 70, 147-157.

Koutsikopoulos C, Lacroix N (1992) Distribution and abundance of sole (*Solea solea* (L)) eggs and larvae in the Bay of Biscay between 1986 and 1989. Netherlands Journal of Sea Research, 29, 81-91.

Kritzer JP, Sale PF (2004) Metapopulation ecology in the sea: from Levins' model to marine ecology and fisheries science. Fish and Fisheries, 5, 131-140.

Kuhl FP, Giardina CR (1982) Elliptic Fourier Features of a closed contour. Computer Graphics and Image Processing, 18, 236-258.

Kuparinen A, Merilä J (2007) Detecting and managing fisheries-induced evolution. Trends in Ecology & Evolution, 22, 652-659.

L'Abée-Lund JH (1988) Otolith shape discriminates between juvenile Atlantic salmon, *Salmo salar* L., and brown trout, *Salmo trutta* L. Journal of Fish Biology, 33, 899-903.

Lacroix G, Ruddick K, Ozer J, Lancelot C (2004) Modelling the impact of the Scheldt and Rhine/Meuse plumes on the salinity distribution in Belgian waters (southern North Sea). Journal of Sea Research, 52, 149-163.

Lagardère JP (1987). Feeding ecology and daily food consumption of common sole, *Solea vulgaris* Quensel,

juveniles on the French Atlantic coast. Journal of Fish Biology, 30, 91-104.
Lagardère F, Troadec H (1997) Age estimation in common sole *Solea solea* larvae: validation of daily increments and evaluation of a pattern recognition technique. Marine Ecology Progress Series, 155, 223-237.
Lage C, Kornfield I (2006) Reduced genetic diversity and effective population size in an endangered Atlantic salmon (*Salmo salar*) population from Maine, USA. Conservation Genetics, 7, 91-104.
Larmuseau MHD, Huyse T, Vancampenhout K, Van Houdt JKJ, Volckaert FAM (2010a) High molecular diversity in the rhodopsin gene in closely related goby fishes: A role for visual pigments in adaptive speciation? Molecular Phylogenetics and Evolution, 55, 689-698.
Larmuseau MHD, Raeymaekers JAM, Hellemans B, Van Houdt JKJ, Volckaert FAM (2010b) Mito-nuclear discordance in the degree of population differentiation in a marine goby. Heredity, 105, 532-542.
Larmuseau MHD, Raeymaekers JAM, Ruddick KG, Van Houdt JKJ, Volckaert FAM (2009) To see in different seas: spatial variation in the rhodopsin gene of the sand goby (*Pomatoschistus minutus*). Molecular Ecology, 18, 4227-4239.
Larsen PF, Nielsen EE, Williams TD, Hemmer-Hansen J, Chipman JK, Kruhoffer M, Gronkjaer P, George SG, Dyrskjot L, Loeschcke V (2007) Adaptive differences in gene expression in European flounder (*Platichthys flesus*). Molecular Ecology, 16, 4674-4683.
Larson RJ, Julian RM (1999) Spatial and temporal genetic patchiness in marine populations and their implications for fisheries management. CALCOFI Report, 40, 94-99.
Larsson LC, Laikre L, Andre C, Dahlgren TG, Ryman N (2010) Temporally stable genetic structure of heavily exploited Atlantic herring (*Clupea harengus*) in Swedish waters. Heredity, 104, 40-51.
Larsson LC, Laikre L, Palm S, André C, Carvalho GR, Ryman N (2007) Concordance of allozyme and microsatellite differentiation in a marine fish, but evidence of selection at a microsatellite locus. Molecular Ecology, 16, 1135-1147.
Law R (2000) Fishing, selection, and phenotypic evolution. ICES Journal of Marine Science, 57, 659-668.
Law R (2007) Fisheries-induced evolution: present status and future directions. Marine Ecology-Progress Series, 335, 271-277.
Le Pape O, Baulier L, Cloarec A, Martin J, Le Loc'h F, Desaunay Y (2007) Habitat suitability for juvenile common sole (*Solea solea,* L.) in the Bay of Biscay (France): A quantitative description using indicators based on epibenthic fauna. Journal of Sea Research, 57, 126-136.
Le Pape O, Chauvet F, Desaunay Y, Guerault D (2003a) Relationship between interannual variations of the river plume and the extent of nursery grounds for the common sole (*Solea Solea*, L.) in Vilaine Bay. Effects on recruitment variability. Journal of Sea Research, 50, 177-185.
Le Pape O, Holley J, Guerault D, Desaunay Y (2003b) Quality of coastal and estuarine essential fish habitats: estimations based on the size of juvenile common sole (*Solea Solea* L.). Estuarine Coastal and Shelf Science, 58, 793-803.
Leakey CDB, Attrill MJ, Fitzsimons MF (2009) Multi-element otolith chemistry of juvenile sole (*Solea solea*), whiting (*Merlangius merlangus*) and European seabass (*Dicentrarchus labrax)* in the Thames Estuary and adjacent coastal regions. Journal of Sea Research, 61, 268-274.
Leakey CDB, Attrill MJ, Jennings S, Fitzsimons MF (2008) Stable isotopes in juvenile marine fishes and their invertebrate prey from the Thames Estuary, UK, and adjacent coastal regions. Estuarine Coastal and Shelf Science, 77, 513-522.
Lescrauwaet A-K, Debergh H, Vincx M, Mees J (2010). Fishing in the past: Historical data on sea fisheries landings in Belgium, Marine Policy, 34, 1279-1289.
Leonard JA (2008) Ancient DNA applications for wildlife conservation. Molecular Ecology, 17, 4186-4196.
Levins R (1970) Extinction. In: Some Mathematical Problems in Biology (ed. M. Desternhaber). American Mathematical Society, Providence, 77–107.
Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25, 1451-1452.
Limborg MT, Pedersen JS, Hemmer-Hansen J, Tomkiewicz J, Bekkevold D (2009) Genetic population structure of European sprat *Sprattus sprattus*: differentiation across a steep environmental gradient in a small pelagic fish. Marine Ecology-Progress Series, 379, 213-224.
Limburg KE (1995) Otolith strontium traces environmental history of subyearling American shad *Alosa sapidissima*. Marine Ecology-Progress Series, 119, 25-35.
Lleonart J, Salat J, Torres GJ (2000) Removing allometric effects of body size in morphological analysis. Journal of Theoretical Biology, 205, 85-93.

Longerich HP, Jackson SE, Gunther D (1996) Laser ablation inductively coupled plasma mass spectrometric transient signal data acquisition and analyte concentration calculation. Journal of Analytical Atomic Spectrometry, 11, 899-904.

Longmore C, Fogarty K, Neat F, Brophy D, Trueman C, Milton A, Mariani S (2010) A comparison of otolith microchemistry and otolith shape analysis for the study of spatial variation in a deep-sea teleost, *Coryphaenoides rupestris*. Environmental Biology of Fishes, 89, 591-605.

Lucentini L, Caporali S, Palomba A, Lancioni H, Panara F (2006) A comparison of conservative DNA extraction methods from fins and scales of freshwater fish: A useful tool for conservation genetics. Conservation Genetics, 7, 1009-1012.

Luikart G, Allendorf FW, Cornuet JM, Sherwin WB (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. Journal of Heredity, 89, 238-247.

Luikart G, Ryman N, Tallmon D, Schwartz M, Allendorf F (2010) Estimation of census and effective population sizes: the increasing usefulness of DNA-based approaches. Conservation Genetics, 11, 355-373.

Lukoschek V, Waycott M, Keogh JS (2008) Relative information content of polymorphic microsatellites and mitochondrial DNA for inferring dispersal and population genetic structure in the olive sea snake, *Aipysurus laevis*. Molecular Ecology, 17, 3062-3077.

Lundy CJ, Moran P, Rico C, Milner RS, Hewitt GM (1999) Macrogeographical population differentiation in oceanic environments: a case study of European hake (*Merluccius merluccius*), a commercially important fish. Molecular Ecology, 8, 1889-1898.

MacKenzie K, Campbell N, Mattiucci S, Ramos P, Pinto AL, Abaunza P (2008) Parasites as biological tags for stock identification of Atlantic horse mackerel *Trachurus trachurus* L. Fisheries Research, 89, 136-145.

Manel S, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. Trends in Ecology & Evolution, 18, 189-197.

Mantel N (1967) Detection of disease clustering and a generalized regression approach. Cancer Research, 27, 209-220.

Mariani S, Hutchinson WF, Hatfield EMC, Ruzzante DE, Simmonds EJ, Dahlgren TG, Andre C, Brigham J, Torstensen E, Carvalho GR (2005) North Sea herring population structure revealed by microsatellite analysis. Marine Ecology-Progress Series, 303, 245-257.

Martinsohn JT, Geffen AJ, Maes GE, Nielsen EE, Ogden R, Waples RS, Carvalho GR, Consortium TF (In Press) From ocean to fork: Traceability of fish and fish products based on advanced molecular technologies.

Matschiner M, Salzburger W (2009) TANDEM: integrating automated allele binning into genetics and genomics workflows. Bioinformatics, 25, 1982-1983.

Maudet C, Miller C, Bassano B, Breitenmoser-Wursten C, Gauthier D, Obexer-Ruff G, Michallet J, Taberlet P, Luikart G (2002) Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in Alpine ibex (*Capra ibex* (ibex)). Molecular Ecology, 11, 421-436.

McCusker MR, Bentzen P (2010) Positive relationships between genetic diversity and abundance in fishes. Molecular Ecology, 19, 4852-4862.

McQuinn IH (1997) Metapopulations and the Atlantic herring. Reviews in Fish Biology and Fisheries, 7, 297-329.

Mérigot B, Letourneur Y, Lecomte-Finiger R (2007) Characterization of local populations of the common sole *Solea solea* (Pisces, Soleidae) in the NW Mediterranean through otolith morphometrics and shape analysis. Marine Biology, 151, 997-1008.

Middelburg JJ, Levin LA (2009). Coastal hypoxia and sediment biogeochemistry, Biogeosciences, 6, 1273-1293.

Millar RB (1990a) Comparison of methods for estimating mixed stock fishery composition. Canadian Journal of Fisheries and Aquatic Sciences, 47, 2235-2241.

Millar RB (1990b) A versatile computer program for mixed stock fishery composition estimation. Canadian Technical Report of Fisheries and Aquatic Sciences, No. 1753, iii + 29 pp.

Miller JA, Banks MA, Gomez-Uchida D, Shanks AL (2005) A comparison of population structure in black rockfish (*Sebastes melanops*) as determined with otolith microchemistry and microsatellite DNA. Canadian Journal of Fisheries and Aquatic Sciences, 62, 2189-2198.

Miller JA, Bellinger MR, Golden JT, Fujishin L, Banks MA (2010) Integration of natural and artificial markers in a mixed stock analysis of Chinook salmon (*Oncorhynchus tshawytscha*). Fisheries Research, 102, 152-159.

Miller LM, Kapuscinski AR (1997) Historical analysis of genetic variation reveals low effective population size in a northern pike (*Esox lucius*) population. Genetics, 147, 1249-1258.

Millner R, Whiting C (1996) Long-term changes in growth and population abundance of sole in the North Sea from 1940 to the present. ICES Journal of Marine Science, 53, 1185-1195.

Mills SL (2007) Conservation of wildlife populations. Blackwell Publishing, Oxford, UK. 424pp.

Molinero A, Flos R (1991) Influence of sex and age on the feeding habits of the common sole *Solea solea*. Marine Biology, 111, 493-501.

Molinero A, Flos R (1992) Influence of season on the feeding habits of the common sole *Solea solea*. Marine Biology, 113, 499-507.

Mollet FM, Kraak SBM, Rijnsdorp AD (2007) Fisheries-induced evolutionary changes in maturation reaction norms in North Sea sole *Solea solea*. Marine Ecology-Progress Series, 351, 189-199.

Morita K, Fukuwaka M (2007) Why age and size at maturity have changed in Pacific salmon. Marine Ecology-Progress Series, 335, 289-294.

Mullon C, Freon P, Cury P (2005) The dynamics of collapse in world fisheries. Fish and Fisheries, 6, 111-120.

Myers RA, Worm B (2003) Rapid worldwide depletion of predatory fish communities. Nature, 423, 280-283.

Nei M (1973) Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences, USA, 70, 3321–3323.

Nesbø CL, Rueness EK, Iversen SA, Skagen DW, Jakobsen KS (2000) Phylogeography and population history of Atlantic mackerel (*Scomber scombrus* L.): a genealogical approach reveals genetic structuring among the eastern Atlantic stocks. Proceedings of the Royal Society of London Series B-Biological Sciences, 267, 281-292.

Nielsen EE, Hansen MM (2008) Waking the dead: the value of population genetic analyses of historical samples. Fish and Fisheries, 9, 450-461.

Nielsen EE, Hansen MM, Loeschcke V (1997) Analysis of microsatellite DNA from old scale samples of Atlantic salmon *Salmo salar*: A comparison of genetic composition over 60 years. Molecular Ecology, 6, 487-492.

Nielsen EE, Hansen MM, Loeschcke V (1999) Analysis of DNA from old scale samples: technical aspects, applications and perspectives for conservation. Hereditas, 130, 265-276.

Nielsen EE, Hansen MM, Ruzzante DE, Meldrup D, Gronkjaer P (2003) Evidence of a hybrid-zone in Atlantic cod (*Gadus morhua*) in the Baltic and the Danish Belt Sea revealed by individual admixture analysis. Molecular Ecology, 12, 1497-1508.

Nielsen EE, Hansen MM, Schmidt C, Meldrup D, Gronkjaer P (2001) Fisheries - Population of origin of Atlantic cod. Nature, 413, 272-272.

Nielsen EE, Hemmer-Hansen J, Larsen PF, Bekkevold D (2009a) Population genomics of marine fishes: identifying adaptive variation in space and time. Molecular Ecology, 18, 3128-3150.

Nielsen EE, Hemmer-Hansen J, Poulsen NA, Loeschcke V, Moen T, Johansen T, Mittelholzer C, Taranger GL, Ogden R, Carvalho GR (2009b) Genomic signatures of local directional selection in a high gene flow marine organism; the Atlantic cod (*Gadus morhua*). BMC Evolutionary Biology, 9, 276-287.

Nielsen EE, Nielsen PH, Meldrup D, Hansen MM (2004) Genetic population structure of turbot (*Scophthalmus maximus* L.) supports the presence of multiple hybrid zones for marine fishes in the transition zone between the Baltic Sea and the North Sea. Molecular Ecology, 13, 585-595.

Nolting RF (1986) Copper, Zinc, Cadmium, Nickel, Iron and Manganese in the Southern Bight of the North-Sea. Marine Pollution Bulletin, 17, 113-117.

O'Connell M, Wright JM (1997) Microsatellite DNA in fishes. Reviews in Fish Biology and Fisheries, 7, 331-363.

O'Reilly PT, Canino MF, Bailey KM, Bentzen P (2004) Inverse relationship between F_{ST} and microsatellite polymorphism in the marine fish, walleye pollock (*Theragra chalcogramma*): implications for resolving weak population structure. Molecular Ecology, 13, 1799-1814.

Ogden R (2008) Fisheries forensics: the use of DNA tools for improving compliance, traceability and enforcement in the fishing industry. Fish and Fisheries, 9, 462-472.

Oksanen J, Kindt R, Legendre P, O'Hara RB (2007) Community Ecology Package version 1.8-5: vegan, http://cran.r-project.org/.

OSPAR (2000) Quality Status Report 2000. OSPAR Commission London, 108 + vii pp.

OSPAR (2005) Assessment of data collected under the OSPAR comprehensive study on riverine inputs and direct discharges for the period 1990-2002. OSPAR Commission, publication number 2005/233.

OSPAR (2010) Quality Status Report 2010. OSPAR Commission London, 176 pp.

Otto L, Zimmerman JTF, Furnes GK, Mork M, Saetre R, Becker G (1990) Review of the physical oceanography of the North Sea. Netherlands Journal of Sea Research, 26, 161-238.

Paabo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, Hofreiter M (2004) Genetic analyses from ancient DNA. Annual Review of Genetics, 38, 645-679.

Palsbøll PJ, Berube M, Allendorf FW (2007) Identification of management units using population genetic data. Trends in Ecology & Evolution, 22, 11-16.

Palstra FP, Ruzzante DE (2008) Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? Molecular Ecology, 17, 3428-3447.

Palumbi SR (2003) Population genetics, demographic connectivity, and the design of marine reserves. Ecological Applications, 13, 146-158.

Palumbi SR (2004a) Fisheries Science - Why mothers matter. Nature, 430, 621-622.

Palumbi SR (2004b) Marine reserves and ocean neighborhoods: the spatial scale of marine populations and their management. Annual Review of Environment and Resources, 29, 31-68.

Pampoulie C, Gysels ES, Maes GE, Hellemans B, Leentjes V, Jones AG, Volckaert FAM (2004) Evidence for fine-scale genetic structure and estuarine colonisation in a potential high gene flow marine goby (*Pomatoschistus minutus*). Heredity, 92, 434-445.

Panfili J, de Pontual H, Troadec H, Wright PJ (2002) Manual of fish sclerochronology Ifremer-IRD coedition, Brest, France. 464 pp.

Patarnello T, Volckaert FAM, Castilho R (2007) Pillars of Hercules: is the Atlantic-Mediterranean transition a phylogeographical break? Molecular Ecology, 16, 4426-4444.

Patterson WF, Cowan JH, Wilson CA, Chen ZX (2008) Temporal and spatial variability in juvenile red snapper otolith elemental signatures in the northern Gulf of Mexico. Transactions of the American Fisheries Society, 137, 521-532.

Patterson HM, McBride RS, Julien N (2004) Population structure of red drum (*Sciaenops ocellatus*) as determined by otolith chemistry. Marine Biology, 144, 855-862.

Pauly D, Christensen V, Dalsgaard J, Froese R, Torres F, Jr. (1998) Fishing Down Marine Food Webs. Science, 279, 860-863.

Payan P, De Pontual H, Boeuf G, Mayer-Gostan N (2004) Endolymph chemistry and otolith growth in fish. Comptes Rendus Palevol, 3, 535-547.

Pawson MG (1995) Biogeographical identification of English Channel fish and shellfish stocks. (ed. Fisheries Research Technical Report No. 99. Ministry of Agriculture FaF), Directorate of Fisheries Research, Lowestoft. 72pp.

Pearse DE, Crandall KA (2004) Beyond Fst: Analysis of population genetic data for conservation. Conservation Genetics, 5, 585-602.

Pinnegar J, Engelhard G (2008) The 'shifting baseline' phenomenon: a global perspective. Reviews in Fish Biology and Fisheries, 18, 1-16.

Piry S, Alapetite A, Cornuet JM, Paetkau D, Baudouin L, Estoup A (2004) GENECLASS2: A software for genetic assignment and first-generation migrant detection. Journal of Heredity, 95, 536-539.

Porta J, Alvarez MC (2004) Development and characterization of microsatellites from Senegal sole (*Solea senegalensis*). Molecular Ecology Notes, 4, 277-279.

Portnoy DS, McDowell JR, Heist EJ, Musick JA, Graves JE (2010) World phylogeography and male-mediated gene flow in the sandbar shark, *Carcharhinus plumbeus*. Molecular Ecology, 19, 1994-2010.

Poulsen NA, Nielsen EE, Schierup MH, Loeschcke V, Gronkjaer P (2006) Long-term stability and effective population size in North Sea and Baltic Sea cod (*Gadus morhua*). Molecular Ecology, 15, 321-331.

Power M, Attrill MJ, Thomas RM (1999) Heavy metal concentration trends in the Thames estuary. Water Research, 33, 1672-1680.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics, 155, 945-959.

Pujolar JM, Maes GE, Volckaert FAM (2006) Genetic patchiness among recruits in the European eel *Anguilla anguilla*. Marine Ecology-Progress Series, 307, 209-217.

Rabaut M (2009) *Lanice conchilega*, fisheries and marine conservation: Towards an ecosystem approach to marine management. PhD thesis, Ghent University (UGENT). 398 pp.

Rabaut M, Braeckman U, Hendrickx F, Vincx M, Degraer S (2008) Experimental beam-trawling in *Lanice conchilega* reefs: Impact on the associated fauna. Fisheries Research, 90, 209-216.

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. Proceedings of the National Academy of Sciences of the United States of America, 94, 9197-9201.

Rasband WS (1997-2009) ImageJ eds. U. S. National Institutes of Health B, Maryland U).

Raymond M, Rousset F (1995) Genepop (Version-1.2) - Population genetics software for exact tests and ecumenicism. Journal of Heredity, 86, 248-249.

Reed DH, Frankham R (2001) How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. Evolution, 55, 1095-1103.

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. Conservation Biology, 17, 230-237.

Reiss H, Hoarau G, Dickey-Collas M, Wolff WJ (2009) Genetic population structure of marine fish: mismatch between biological and fisheries management units. Fish and Fisheries, 10, 361-395.

Rice WR (1989) Analyzing tables of statistical tests. Evolution, 43, 223-225.

Rijnsdorp AD, Grift RE, Kraak SBM (2005) Fisheries-induced adaptive change in reproductive investment in North Sea plaice (*Pleuronectes platessa*)? Canadian Journal of Fisheries and Aquatic Sciences, 62, 833-843

Rijnsdorp AD, Poos JJ, Quirijns FJ, Hille Ris Lambers R, De Wilde JW, Den Heijer WM (2008) The arms race between fishers. Journal of Sea Research, 60, 126-138.

Rijnsdorp AD, van Beek FA, Flatman S, Millner RM, Riley JD, Giret M, Declerck R (1992) Recruitment of sole stocks, *Solea solea* (L) in the Northeast Atlantic. Netherlands Journal of Sea Research, 29, 173-192.

Rijnsdorp AD, Vingerhoed B (1994) The ecological significance of geographical and seasonal differences in egg size in sole *Solea solea* (L.). Netherlands Journal of Sea Research, 32, 255-270.

Rijnsdorp AD, Witthames PR (2004) Ecology of reproduction. In: Flatfishes. Biology and exploitation. Ed. Gibson RN. Blackwell Publishing, Oxford, UK, p. 68-87.

Rogers SI, Millner RS, Mead TA (1998) The distribution and abundance of young fish on the east and south coast of England (1981 to 1997). Science Series Technical Report, CEFAS, Lowestoft, 108, 133 pp.

Rodhe J (1996) On the dynamics of the large-scale circulation of the Skagerrak. Journal of Sea Research, 35, 9-21.

Rolland JL, Bonhomme F, Lagardere F, Hassan M, Guinand B (2007) Population structure of the common sole (*Solea solea*) in the Northeastern Atlantic and the Mediterranean Sea: revisiting the divide with EPIC markers. Marine Biology, 151, 327-341.

Rooker JR, Secor DH, De Metrio G, Schloesser R, Block BA, Neilson JD (2008) Natal homing and connectivity in Atlantic Bluefin tuna populations. Science, 322, 742-744.

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes, 4, 137-138.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. Genetics, 145, 1219-1228.

Russell FS (1976) The eggs and planktonic stages of British marine fishes. Academic Press, London, 524 pp.

Ruttenberg BI, Hamilton SL, Hickford MJH, Paradis GL, Sheehy MS, Standish JD, Ben-Tzvi O, Warner RR (2005) Elevated levels of trace elements in cores of otoliths and their potential for use as natural tags. Marine Ecology-Progress Series, 297, 273-281.

Ruzzante DE (2001) Stability in the historical pattern of genetic structure of Newfoundland cod (*Gadus morhua*) despite the catastrophic decline in population size from 1964 to 1994. Conservation genetics, 2, 257-269.

Ruzzante DE, Mariani S, Bekkevold D, Andre C, Mosegaard H, Clausen LAW, Dahlgren TG, Hutchinson WF, Hatfield EMC, Torstensen E, Brigham J, Simmonds EJ, Laikre L, Larsson LC, Stet RJM, Ryman N, Carvalho GR (2006) Biocomplexity in a highly migratory pelagic marine fish, Atlantic herring. Proceedings of the Royal Society B-Biological Sciences, 273, 1459-1464.

Ryman N, Palm S (2006) POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. Molecular Ecology Notes, 6, 600-602.

Saisa M, Koljonen ML, Tahtinen J (2003) Genetic changes in Atlantic salmon stocks since historical times and the effective population size of a long-term captive breeding programme. Conservation Genetics, 4, 613-627.

Sala-Bozano M, Ketmaier V, Mariani S (2009) Contrasting signals from multiple markers illuminate population connectivity in a marine fish. Molecular Ecology, 18, 4811-4826.

Sambrook J, Russell D (2001). Molecular cloning: A laboratory manual, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Savina M, Lacroix G, Ruddick K (2010) Modelling the transport of common sole larvae in the southern North Sea: Influence of hydrodynamics and larval vertical movements. Journal of Marine Systems, 81, 86-98.

Schaffler JJ, Winkelman DL (2008) Temporal and spatial variability in otolith trace-element signatures of juvenile striped bass from spawning locations in Lake Texoma, Oklahoma-Texas. Transactions of the American Fisheries Society, 137, 818-829.

Schloesser RW, Neilson JD, Secor DH, Rooker JR (2010) Natal origin of Atlantic bluefin tuna *(Thunnus thynnus)* from Canadian waters based on otolith delta C-13 and delta O-18. Canadian Journal of Fisheries and Aquatic Sciences, 67, 563-569.

Scholten MCT, Kramer KJM, Laane R (1998) Trends and variation in concentration of dissolved metals (Cd, Cu, Pb, and Zn) in the North Sea (1980-1989). ICES Journal of Marine Science, 55, 825-834.

Secor DH (1999) Specifying divergent migrations in the concept of stock: the contingent hypothesis. Fisheries Research, 43, 13-34.

Secor DH, Kerr LA, Cadrin SX (2009) Connectivity effects on productivity, stability, and persistence in a herring metapopulation model. ICES Journal of Marine Science, 66, 1726–1732.

Secor DH, Rooker JR (2000) Is otolith strontium a useful scalar of life cycles in estuarine fishes? Fisheries Research, 46, 359-371.

Selkoe KA, Henzler CM, Gaines SD (2008) Seascape genetics and the spatial ecology of marine populations. Fish and Fisheries, 9, 363-377.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters, 9, 615-629.

Selkoe KA, Watson JR, White C, Ben Horin T, Iacchei M, Mitarai S, Siegel DA, Gaines SD, Toonen RJ (2010) Taking the chaos out of genetic patchiness: seascape genetics reveals ecological and oceanographic drivers of genetic patterns in three temperate reef species. Molecular Ecology, 19, 3708-3726.

Shiao JC, Tzeng CS, Leu CL, Chen FC (1999) Enhancing the contrast and visibility of daily growth increments in fish otoliths etched by proteinase K buffer. Journal of Fish Biology, 54, 302-309.

Slatkin M (1993) Isolation by distance in equilibrium and nonequilibrium populations. Evolution, 47, 264-279.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics, 139, 457-462.

Sponaugle S, Cowen RK, Shanks A, Morgan SG, Leis JM, Pineda JS, Boehlert GW, Kingsford MJ, Lindeman KC, Grimes C, Munro JL (2002) Predicting self-recruitment in marine populations: Biophysical correlates and mechanisms. Bulletin of Marine Science, 70, 341-375.

Stefansson G, Rosenberg AA (2006) Designing marine protected areas for migrating fish stocks. Journal of Fish Biology, 69, 66-78.

Stenseth NC, Jorde PE, Chan KS, Hansen E, Knutsen H, Andre C, Skogen MD, Lekve K (2006) Ecological and genetic impact of Atlantic cod larval drift in the Skagerrak. Proceedings of the Royal Society B-Biological Sciences, 273, 1085-1092.

Stokstad E (2010). To fight illegal fishing, forensic DNA gets local. Science, 330, 1468-1469.

Stransky C, Baumann H, Fevolden S-E, Harbitz A, Høie H, Nedreaas KH, Salberg A-B, Skarstein TH (2008) Separation of Norwegian coastal cod and Northeast Arctic cod by outer otolith shape analysis. Fisheries Research, 90, 26-35.

Swan SC, Geffen AJ, Morales-Nin B, Gordon JDM, Shimmield T, Sawyer T, Massuti E (2006) Otolith chemistry: an aid to stock separation of *Helicolenus dactylopterus* (bluemouth) and *Merluccius merluccius* (European hake) in the Northeast Atlantic and Mediterranean. ICES Journal of Marine Science, 63, 504-513.

Swearer SE, Shima JS, Hellberg ME, Thorrold SR, Jones GP, Robertson DR, Morgan SG, Selkoe KA, Ruiz GM, Warner RR (2002) Evidence of self-recruitment in demersal marine populations. Bulletin of Marine Science, 70, 251-271.

Swearer SE, Forrester GE, Steele MA, Brooks AJ, Lea DW (2003) Spatio-temporal and interspecific variation in otolith trace-elemental fingerprints in a temperate estuarine fish assemblage. Estuarine Coastal and Shelf Science, 56, 1111-1123.

Symonds DJ, Rogers SI (1995) The Influence of spawning and nursery grounds on the distribution of Sole *Solea solea* (L) in the Irish Sea, Bristol Channel and adjacent areas. Journal of Experimental Marine Biology and Ecology, 190, 243-261.

Tallmon DA, Gregovich D, Waples RS, Baker CS, Jackson J, Taylor BL, Archer E, Martien KK, Allendorf FW, Schwartz MK (2010) When are genetic methods useful for estimating contemporary abundance and detecting population trends? Molecular Ecology Resources, 10, 684-692.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10, 512-526.

Teal LR, de Leeuw JJ, van der Veer HW, Rijnsdorp AD (2008) Effects of climate change on growth of 0-group sole and plaice. Marine Ecology-Progress Series, 358, 219-230.

Tessier N, Bernatchez L (1999) Stability of population structure and genetic diversity across generations assessed by microsatellites among sympatric populations of landlocked Atlantic salmon (*Salmo salar* L.). Molecular Ecology, 8, 169-179.

Therkildsen NO, Nielsen EE, Swain DP, Pedersen JS (2010) Large effective population size and temporal genetic stability in Atlantic cod (*Gadus morhua*) in the southern Gulf of St. Lawrence. Canadian Journal of Fisheries and Aquatic Sciences, 67, 1585-1595.

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bulletin of Marine Science, 70, 291-308.

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in a marine fish metapopulation. Science, 291, 297-299.

Thorrold SR, Zacherl DC, Levin LA (2007) Population connectivity and larval dispersal using geochemical signatures in calcified structures. Oceanography, 20, 80-89.

Thresher RE (1999) Elemental composition of otoliths as a stock delineator in fishes. Fisheries Research, 43, 165-204.

Tobin D, Wright PJ, Gibb FM, Gibb IM (2010) The importance of life stage to population connectivity in whiting (*Merlangius merlangus*) from the northern European shelf. Marine Biology, 157, 1063-1073.

Turan C (2004) Stock identification of Mediterranean horse mackerel (*Trachurus mediterraneus*) using morphometric and meristic characters. ICES Journal of Marine Science, 61, 774-781.

Turan C (2006) The use of otolith shape and chemistry to determine stock structure of Mediterranean horse mackerel *Trachurus mediterraneus* (Steindachner). Journal of Fish Biology, 69, 165-180.

Turner TF, Wares JP, Gold JR (2002) Genetic effective size is three orders of magnitude smaller than adult census size in an abundant, estuarine-dependent marine fish (*Sciaenops ocellatus*). Genetics, 162, 1329-1339.

Tuset VM, Lozano IJ, Gonzalez JA, Pertusa JF, Garcia-Diaz MM (2003) Shape indices to identify regional differences in otolith morphology of comber, *Serranus cabrilla* (L., 1758). Journal of Applied Ichthyology, 19, 88-93.

Tuset VM, Rosin PL, Lombarte A (2006) Sagittal otolith shape used in the identification of fishes of the genus Serranus. Fisheries Research, 81, 316-325.

Van Achterberg E, Ryan CG, Jackson S, Griffin WL (2001) Data reduction software for LA- ICP-MS. In: Laser-Ablation-ICPMS in the Earth Sciences, Principles and Applications Short Course Series, vol. 29 (ed. Sylvester P), pp. 239-243. Mineralogical Association of Canada.

van Beek F, Vingerhoed B, Schelvis D (1997). Development of a prototype otolith line for routine age reading of demersal species (OTOLIN). RIVO-DLO Report C027/97. Netherlands Institute for Fisheries Research, The Netherlands.

Van Damme S, Struyf E, Maris T, Ysebaert T, Dehairs F, Tackx M, Heip C, Meire P (2005) Spatial and temporal patterns of water quality along the estuarine salinity gradient of the Scheldt estuary (Belgium and The Netherlands): results of an integrated monitoring approach. Hydrobiologia, 540, 29-45.

Van der Land MA (1991) Distribution of flatfish eggs in the 1989 egg surveys in the Southeastern North Sea, and mortality of plaice and sole eggs. Netherlands Journal of Sea Research, 27, 277-286.

van der Veer HW, Berghahn R, Miller JM, Rijnsdorp AD (2000) Recruitment in flatfish, with special emphasis on North Atlantic species: Progress made by the Flatfish Symposia. ICES Journal of Marine Science, 57, 202-215.

van der Veer HW, Dapper R, Witte JIJ (2001) The nursery function of the intertidal areas in the western Wadden Sea for 0-group sole *Solea solea* (L.). Journal of Sea Research, 45, 271-279.

van Hal R, Smits K, Rijnsdorp AD (2010) How climate warming impacts the distribution and abundance of two small flatfish species in the North Sea. Journal of Sea Research, 64, 76-84.

van Oosterhout C, Weetman D, Hutchinson WF (2006) Estimation and adjustment of microsatellite null alleles in nonequilibrium populations. Molecular Ecology Notes, 6, 255-256.

van Walraven L, Mollet FM, van Damme CJG, Rijnsdorp AD (2010) Fisheries-induced evolution in growth, maturation and reproductive investment of the sexually dimorphic North Sea plaice (*Pleuronectes platessa* L.). Journal of Sea Research, 64, 85-93.

Vasconcelos RP (2007) Discriminating estuarine nurseries for five fish species through otolith elemental fingerprints. Marine Ecology Progress Series, 350, 117-126.

Vasconcelos RP, Reis-Santos P, Tanner S, Maia A, Latkoczy C, Gunther D, Costa MJ, Cabral H (2008) Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints. Estuarine Coastal and Shelf Science, 79, 317-327.

Vasconcelos RP, Reis-Santos P, Tanner S, Maia A, Latkoczy C, Gunther D, Costa MJ, Cabral H (2009) Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints (vol 79, pg 317, 2008). Estuarine Coastal and Shelf Science, 82, 363-364.

Vignal A, Milan D, SanCristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. Genetics Selection Evolution, 34, 275-305.

Vinagre C, Amara R, Maia A, Cabral HN (2008a) Latitudinal comparison of spawning season and growth of 0-group sole, *Solea solea* (L.). Estuarine Coastal and Shelf Science, 78, 521-528.

Vinagre C, Salgado J, Costa MJ, Cabral HN (2008b) Nursery fidelity, food web interactions and primary sources of nutrition of the juveniles of *Solea solea* and *S. senegalensis* in the Tagus estuary (Portugal): A stable isotope approach. Estuarine Coastal and Shelf Science, 76, 255-264.

Walsh B, Lynch M (in press) The genetic effective size of a populations. In: Evolution and selection of quantitative traits, p. 30.

Walther BD, Thorrold SR, Olney JE (2008) Geochemical signatures in otoliths record natal origins of American shad. Transactions of the American Fisheries Society, 137, 57-69.

Wandeler P, Hoeck PEA, Keller LF (2007) Back to the future: museum specimens in population genetics. Trends in Ecology & Evolution, 22, 634-642.

Wang, JL (2001) A pseudo-likelihood method for estimating effective population size from temporally spaced samples. Genetics Research, 78, 243–257.

Wang J (2005) Estimation of effective population sizes from data on genetic markers. Philosophical Transactions of the Royal Society B: Biological Sciences, 360, 1395-1409.

Wang J, Whitlock MC (2003) Estimating effective population size and migration rates from genetic samples over space and time. Genetics, 163, 429-446.

Waples RS (1989) A generalized approach for estimating effective population size from temporal changes in allele frequency. Genetics, 121, 379-391.

Waples RS (1998) Separating the wheat from the chaff: Patterns of genetic differentiation in high gene flow species. Journal of Heredity, 89, 438-450.

Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conservation Genetics, 7, 167-184.

Waples RS (2010) The state of the field: Spatial-temporal stratifications in natural populations and how they affect understanding and estimation of effective population size. Molecular Ecology Resources, 10, 785-796.

Waples RS, Do C (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. Molecular Ecology Resources, 8, 753-756.

Waples RS, Gaggiotti O (2006) What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. Molecular Ecology, 15, 1419-1439.

Waples RS, Punt AE, Cope JM (2008) Integrating genetic data into management of marine resources: how can we do it better? Fish and Fisheries, 9, 423-449.

Waples RS, Yokota M (2007) Temporal estimates of effective population size in species with overlapping generations. Genetics, 175, 219-233.

Warner RR, Hamilton SL, Sheehy MS, Zeidberg LD, Brady BC, Caselle JE (2009) Geographic variation in natal and early larval trace-elemental signatures in the statoliths of the market squid *Doryteuthis* (formerly Loligo) *opalescens*. Marine Ecology-Progress Series, 379, 109-121.

Warner RR, Swearer SE, Caselle JE, Sheehy M, Paradis G (2005) Natal trace-elemental signatures in the otoliths of an open-coast fish. Limnology and Oceanography, 50, 1529-1542.

Was A, Gosling E, Hoarau G (2010) Microsatellite analysis of plaice (*Pleuronectes platessa* L.) in the NE Atlantic: weak genetic structuring in a milieu of high gene flow. Marine Biology, 157, 447-462.

Watts PC, Nash RDM, Kemp SJ (2004) Genetic structure of juvenile plaice *Pleuronectes platessa* on nursery grounds within the Irish Sea. Journal of Sea Research, 51, 191-197.

Wegner G, Damm U, Purps M (2003) Physical influences on the stock dynamics of plaice and sole in the North Sea. Scientia Marina, 67, 219-234.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution, 38, 1358-1370.

Wenne R, Boudry P, Hemmer-Hansen J, Lubieniecki KP, Was A, Kause A (2007) What role for genomics in fisheries management and aquaculture? Aquatic Living Resources, 20, 241-255.

Werner FE, Cowen RK, Paris CB (2007) Coupled biological and physical models present capabilities and necessary developments for future studies of population connectivity. Oceanography, 20, 54-69.

White C, Selkoe KA, Watson J, Siegel DA, Zacherl DC, Toonen RJ (2010) Ocean currents help explain population genetic structure. Proceedings of the Royal Society B-Biological Sciences, 277, 1685-1694.

White JW, Ruttenberg BI (2007) Discriminant function analysis in marine ecology: some oversights and their solutions. Marine Ecology-Progress Series, 329, 301-305.

White JW, Standish JD, Thorrold SR, Warner RR (2008) Markov Chain Monte Carlo methods for assigning larvae to natal sites using natural geochemical tags. Ecological Applications, 18, 1901-1913.

Whitlock MC, McCauley DE (1999) Indirect measures of gene flow and migration: F_{ST} not equal 1/(4Nm+1). Heredity, 82, 117-125.

Witthames PR, Greer Walker M, Dinis MT, Whiting CL (1995) The geographical variation in the potential annual fecundity of dover sole *Solea solea* (L.) from European shelf waters during 1991. Netherlands Journal of Sea Research, 34, 45-58.

Woodhead PJM (1964a) Changes in the behaviour of the sole, *Solea vulgaris*, during cold winters and the relation between the winter catch and sea temperature. Helgoländer wissenschaftlichen Meeresuntersuchung 10, 328-342.

Woodhead PJM (1964b) The death of North Sea fish during the winter of 1962/63 particularly with reference to the sole, *Solea vulgaris*. Helgoländer wissenschaftlichen Meeresuntersuchung, 10, 283-300.

Wright S (1931) Evolution in Mendelian populations. Genetics, 16, 57-159.

Wright PJ, Neat FC, Gibb FM, Gibb IM, Thordarson H (2006) Evidence for metapopulation structuring in cod from the West of Scotland and North Sea. Journal of Fish Biology, 69, 181-199.

Wright PJ, Tobin D, Gibb FM, Gibb IM (2010) Assessing nursery contribution to recruitment: relevance of closed areas to haddock *Melanogrammus aeglefinus*. Marine Ecology-Progress Series, 400, 221-232.

Wright PJ, Trippel EA (2009) Fishery-induced demographic changes in the timing of spawning: consequences for reproductive success. Fish and Fisheries, 10, 283-304.

PUBLICATION LIST

Peer reviewed publications

- **Cuveliers, E.L.**; Volckaert, F.A.M.; Rijnsdorp, A.D.; Larmuseau, M.H.D. & Maes, G.E. (In revision, *Molecular Ecology*). Temporal genetic stability and high effective population size in the North Sea sole over a 50-year time period coinciding with high fishing pressure.

- **Cuveliers, E.L.** ; Maes, GE. ; Guelinckx, J; Volckaert, FAM & Geffen, AJ (2010). Microchemical variation in juvenile sole (*Solea solea*) otoliths as a powerful tool for studying connectivity in the North Sea. *Marine Ecology Progress Series, 401*: 211–220.

- **Cuveliers, E.L.;** Bolle, L.J.; Volckaert, F.A.M. & Maes, G.E (2009). Influence of DNA isolation from historical otoliths on nuclear-mitochondrial marker amplification and age determination in an overexploited fish, the common sole (*Solea solea* L.). *Molecular Ecology Resources*, 9:725-732.

- Pedersen, T.; **Cuveliers, E.L.** & E. Berg (2008). Spatial scales of movement in Northeast Atlantic coastal cod. *Reviews in Fisheries Science,* 16: 348-356.

National reports

- Vanaverbeke, J.; Braeckman, U.; **Cuveliers, E.**; Courtens, W.; Huyse, T.; Lacroix, G.; Larmuseau, M.H.D; Maes, G.; Provoost, P.; Rabaut, M.; Remerie, T.; Savina, M.; Soetaert, K.; Stienen, E.W.M.; Verstraete, H.; Volckaert, F.; Vincx, M. (2009). WestBanks: Understanding benthic, pelagic and airborne ecosystem interactions in shallow coastal seas. Final report phase 1. Project of the Belgian Federal Office for Scientific, Technical and Cultural Affairs, Contract nr. SD/BN/01A.

- Guelinckx, J., **Cuveliers, E.**, Stevens, M., Ollevier, F., Breine, J., Belpairen, C. (2008) Opvolging van het visbestand van de Zeeschelde: resultaten voor 2007. *Rapporten van het Instituut voor Natuur- en Bosonderzoek*, 2008 (39). Instituut voor Natuur- en Bosonderzoek: Brussel: Belgium. 47 pp.

- **Cuveliers, E.L.**, Stevens, M., Guelinckx J., Ollevier, F., Breine, J., Belpaire, C. (2007). Opvolging van het visbestand van de Zeeschelde: resultaten voor 2006. *Rapporten van het Instituut voor Natuur- en Bosonderzoek*, 2007(48). Instituut voor Natuur- en Bosonderzoek: Brussel: Belgium. 35 pp.

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