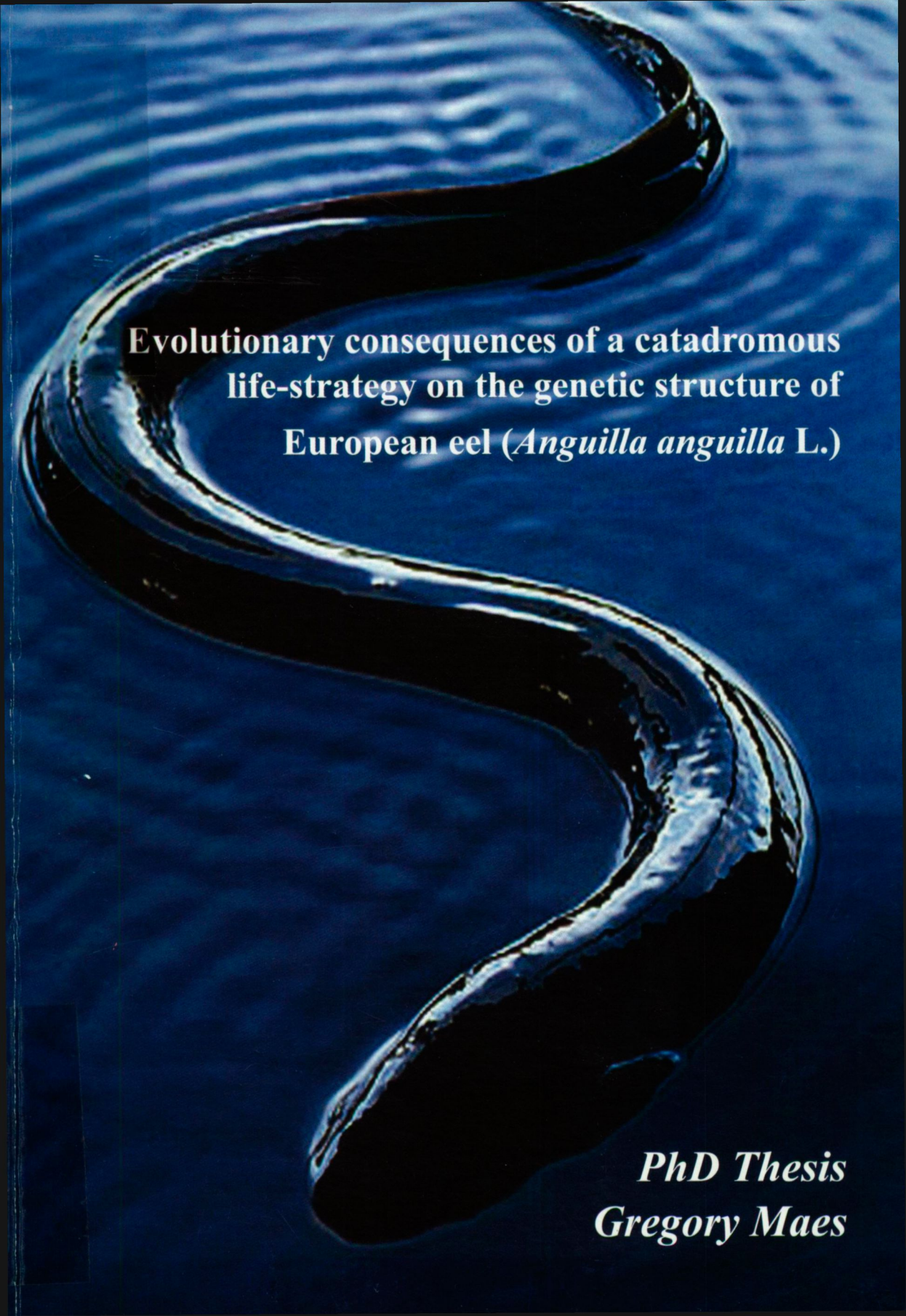
Evolutionary consequences of a catadromous life-strategy on the genetic structure of European eel (*Anguilla anguilla* L.)
PhD Thesis
Gregory Maes

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PhD Thesis

Gregory Maes

2005

FACULTEIT WETENSCHAPPEN
Afdeling Ecologie en Systematiek der Dieren
Laboratorium voor Aquatische Ecologie
Charles Deberiotstraat 32
BE-3000 LEUVEN

KATHOLIEKE
UNIVERSITEIT
LEUVEN

Promotor: Prof. F.A. M. Volckaert
Laboratorium voor Aquatische Ecologie
Katholieke Universiteit Leuven

Overige leden van de examencommissie:

Prof. Dr. T. Wirth
Dr. E. Nielsen
Dr. P. Galbusera
Prof. Dr. L. De Meester
Prof. Dr. F. Ollevier

Het onderzoek ,voorgesteld in dit proefschrift werd gesteund door een beurs verleend door het Vlaams Instituut voor de Bevordering van het Wetenschappelijk–Technologisch Onderzoek in de Industrie (IWT)

KATHOLIEKE UNIVERSITEIT LEUVEN
FACULTEIT WETENSCHAPPEN
DEPARTEMENT BIOLOGIE
Laboratorium voor Aquatische Ecologie

Evolutionary consequences of a catadromous life-strategy on the genetic structure of European eel (*Anguilla anguilla L.*)

Evolutionaire consequenties van een katadrome levens-strategie op de genetische structuur van Europese paling (*Anguilla anguilla* L.)

Promotor :
Prof. Dr. F.A.M. Volckaert

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

Gregory Maes

Insert... break... section... header and footer... Chapter... oh nee, bijna het Dankwoord vergeten ! Eindelijk tijd voor het laatste deel van mijn doctoraat, waar voor één keer géén artikel aan te pas moet komen. Gewoon free writing.

Om dit werk af te krijgen is er heel wat zweet en inkt gevloeid, maar zonder een hele hoop mensen die me daarbij hebben geholpen, zou het wellicht niet gelukt zijn. Van eerste kan "groentje" in 1994 tot doctor in de biologie in 2005 zijn er elf boeiende jaren verstreken ! Het doctoreren is immers de top van de berg (lees : Ardennen), de lange beklimming doe je niet alleen. Waarom ik nu net op die mysterieuze paling wilde doctoreren, stamt al van veel vroeger af. Het begon allemaal in de tweede helft van de jaren tachtig, toen ik elke zondagavond voor de TV zat gekluisterd om naar "Les expéditions du commandant Cousteau" te kijken. In 1988 wilde ik mijn toekomst eens stroomlijnen en schreef ik hem een brief. Tot mijn grote verbazing kreeg ik een persoonlijk antwoord van Cousteau Himself : "De commandant is beroepsmilitair, je kunt maar beter biologie doen !" De keuze was dus snel gemaakt : ik zou Biologie studeren! Na drie jaar studeren wilde ik me specialiseren in de Mariene Biologie, maar dit kon helaas niet in Leuven. Toen ik informeerde om bij Filip Volckaert mijn thesis te maken, volgde echter een openbaring: Mariene vissen bestuderen aan de hand van genetica kon ook! Sindsdien heb ik de smaak te pakken, en na een jaartje buitenland, kon het grote werk beginnen : ik mocht doctoreren!

In de eerste plaats wil ik dan ook mijn promotor Filip Volckaert bedanken voor de kans die hij me gaf om mijn fantasie te laten zegevieren in het uitproberen van genetische testen op paling. Daarnaast zijn er natuurlijk ook nog andere hoofdrolspelers die zonder twijfel tot het welslagen van dit onderzoek hebben bijgedragen. I would like to thank Thierry Wirth, Einar Nielsen, Luc De Meester, Peter Galbusera and Prof. Ollevier for their useful comments on the first version of this PhD thesis. In de tweede plaats wil ik Marti (the PhD-coach) bedanken, zonder wie al deze hoofdstukken niet zo vlug afgewerkt konden worden. De vele "coffee break" gespreken hebben de "eel-team" een nieuw éland gegeven voor de volgende jaren. Vervolgens is er Bart, dé centrale persoon voor al wat met DNA te maken heeft. Bedankt om tot in het oneindige de molariteiten nog maar eens te herhalen en er te zijn geweest bij de menige technische problemen tijdens DNA onderzoek. Geen "groot labo" werk (lees : allozyme werk) zonder de ervaren tussenkomst van Ria, Elke en An W. Nog eens bedankt om op tijd al de stofjes te bestellen. Werken op het laboratorium voor Aquatische Ecologie is meer dan een job, je houdt er (h)echte vriendschappen aan over. Graag wil ik dan ook Jeroen bedanken voor alle"in extremis" hulp bij zovele zaken, zoals mijn kaft, figuren, uitnodigingen en nog zoveel meer. De DNA-groep is altijd een toffe kliek geweest; ik wil dan ook iedereen bedanken voor de vriendschap en hulp: Joost, voor de (ja, alweer) statistische hulp doorheen de jaren en de vele interessante discussies, Els voor het aanleren van allozymes en het verbeteren van mijn "Gregorlands" tijdens mijn thesis; Christophe pour m'avoir montré comment gérer ses résultats (ne pas les laisser reposer, il faut publier !) et pour ton amitié durant tes deux années passées à Leuven (merci à Val aussi !); Nam for the many discussions on STRUCTURE and the great dinners Sokohm made; Franklin and Dima for the useful ideas they gave. En dan nog Kelle voor de vele toffe momenten in en buiten het labo al die jaren lang. Bedankt ook Gerald, Sikke, Geert, Davai voor mijn plezantste jaar aan d' Unif! Tot slot ook een welgemeende "merci" aan Stef, Wouter, Tine, Amal, Eugène, Prissie-Leggie, Sarah, Anton, Jonas, Alessandra, An H., Guy ... Een doctoraat betekent ook thesisstudenten begeleiden. Graag wil ik dan ook Drikke, Dieter, Dirk & Caroke (die na snoek ook de paling is gaan appreciëren) en Tom bedanken voor hun inzet en enthousiasme tijdens de vele gel runs. Jullie gegevens heb ik dan ook met succes kunnen gebruiken in deze thesis.

Naast het palingonderzoek, is conservatiegenetica van zoetwatervissen een tweede passie. Tijdens mijn aanstelling als wetenschappelijke medewerker op snoek en kopvoorn, leerde ik doorheen de jaren

heel wat interessante wetenschappers kennen. Ik wil dan ook heel het personeel van het Instituur voor Bosbouw en Wildbeheer bedanken voor de hulp tijdens staalnames, vooral dan Daniel, Koen, Jan, Gerlinde, Machteld en Claude. Daarnaast ook al de mensen van de Provinciale Visserijcommissies voor hun inzet in het bemonsteren van snoek.

During my repeated sampling trips I learned many, many things from my collaborators, which I could call "international mentors". Special thanks go to: Russell Poole, for his huge effort to sample glass eels yearly. The same is true for Jan Van Willigen (+ Betty) from the RIVO, who helped me since 1999 to get silver and glass eels; Bjarni & Mummi (+ Jun and Prof. Tsukamoto) for the great sampling trips with the 4X4 in Iceland, Johan Dannewitz (& Leif) for the fruitful past and future collaboration. Many other colleagues helped getting samples, namely Pierre Elie, Caroline Durif, Sylvie Dufour, Caroline VanCoillie, Alain Crivelli, Prof. Ahmed Yahyaoui, Dr.Gunnar Naevdahl, Willem Dekker (more for discussions actually), fishermen from Makkum in Friesland, Julian Reynolds, Gail Wippelhauser, Zink Skip and Jun Aoyama. Thanks to the EELREP-group and especially to Guido Van den Thillart forhis guidance.

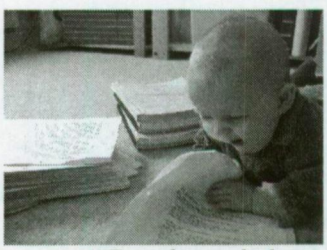
Natuurlijk, een doctoraat is geen 24/24 bezigheid en regelmatig eens op zwier gaan hoort er ook bij. Kelle, Marc, Karen, Joeri, Jeroen, Simon, Koen, Mathias & Jo, Eric & Domya, Peter & Eva, Adi & Sylvie, Wolfgang, Bas, Maarten, Heidi, Charlotte, VFR-Ben : bedankt voor de toffe en ontspannende momenten tijdens de voorbije 11 jaar.

Vrijuit je richting kunnen kiezen en de kansen krijgen om je dromen waar te maken - ook al gebeurt het met vallen en opstaan (jaja, het fenomeen "tweede zit" is mij niet ongekend)- is de verdienste van mijn ouders. Het is niet gemakkelijk om te vertrouwen op een puber die aan de Universiteit begint, maar het is me toch gelukt. Ik wil dan ook mijn familie bedanken voor de jarenlange steun doorheen biologie en het doctoreren, waarbij het niet altijd evident bleek om het belang van palingonderzoek toe te lichten. Mama, papa, Olivier (& Joelle) bedankt voor jullie begrip in soms moeilijke momenten ! Op mijn schoonfamilie kon ik ook altijd rekenen, voor hulp of voor een goed gesprek. Bedankt Marleen (Oppermoesj), Bart & Sofie en Benjamin voor jullie warme onthaal. Net voor het ter perse gaan van dit boek vernam ik het heuglijke nieuws dat ik naast Doctor, ook Peter ben geworden; alvast ook al een dikke merci aan mijn Petekindje Jesse, en aan Jeroen & Sylvie voor hun vertrouwen.

En ten laatste kom ik nog bij mijn eigen kleine familie, Leneke en Amélie. Leneke, het zal voor jou niet altijd gemakkelijk zijn geweest om met een "workaholic" te leven de voorbije jaren. In een doctoraat kan men zich eindeloos verdiepen, wat ik (onbewust) waarschijnlijk iets teveel deed. Al die jaren ben je mij blijven steunen in goede en moeilijke momenten en hebben we samen een toekomst opgebouwd. Met ons Amélietje heb je mij kunnen tonen hoe je echt om iemand kunt geven en hoe onvoorwaardelijk liefhebben voelt. Nu komt een tijd van rust (hum hum...) of tenminste een periode van bezinning van waar het leven écht om draait, namelijk "gelukkig zijn", samen met de mensen rondom mij. En zoals Amélie het zo duidelijk toont : Het leven is meer dan wetenschap alleen !

Bedankt allemaal !

Greg
25/05/2005



Zo vader, zo dochter...

CONTENT

SERVICES ET PRODUITS

- Documentation sur la gestion de l'environnement marin et côtier
- Tables de bases de données océanographiques
- Accès à l'internet
- Référentiel de la zone côtière
- Documentaires : Usages côtiers et Découverte de 'Océan
- CD - Rom images et chiffres de la zone côtière
- Publications et Notes réunies sur l'environnement marin et côtier : série Morphologie, Flore et Faune, Climat, Océanographie…

DOCUMENTATION

- Fonctions : collecter, classer et cataloguer les documents à l'aide du logiciel INMAGIC afin de rendre disponible sur place toutes sortes d'informations océanographiques
- Documents de diverses sources traitant particulièrement du domaine de l'environnement marin et côtier : journaux, publications scientifiques, documents de projets régionaux et inter régionaux (ODINAFRICA II, LME, etc), rapports d'activités d'organismes internationaux (COI, PNUE, PNUD, ONUDI, etc.)
- 522 documents catalogués
- Accès au public, aux institutions académiques et de recherche, aux structures impliquées dans la gestion de l'environnement marin et côtier, aux Organisations Non Gouvernementales

PERSPECTIVES

- Renforcer la production de données et de l'information
- Créer un format de base de données et d'information accessible
- Développer et améliorer les services aux usagers
- Améliorer les relations avec les services producteurs de données par leur organisation et leur traitement afin de rendre l'information utile
- Contribuer au développement des programmes et projets régionaux : Large Marine Ecosystem et Gazoduc de l'Afrique de l'Ouest

Base de données sur les ressources naturelles côtières : Destruction de la mangrove

Contact :
Centre National de Données Océanographiques
Université de Lomé
B.P. 1515 Lomé Togo - Tél : (228) 221 68 17/ (228) 222 48 65
Fax : (228) 221 85 95
Email : a.blivi@odinafrica.net

CENTRE NATIONAL DE DONNEES OCEANOGRAPHIQUES TOGO

L'environnement marin et côtier est un milieu fragile, vulnérable aux pressions anthropiques. La constitution de bases de données par le CNDO en partenariat avec les différentes institutions nationales est une garantie pour le développement durable du secteur.

- Installation officielle : 28 mars 2001
- Siège : **C**entre de **G**estion **I**ntégrée du **L**ittoral et de l'**E**nvironnement de l'Université de Lomé (CGILE/UL)

MISSIONS

- Assurer le service public de données et de l'information scientifique, techniques marines au bénéfice de tous les usagers de l'océan et de la zone côtière
- Rendre efficace le réseau national par le partage de l'expérience sur l'environnement marin et côtier et tenir la responsabilité des échanges, de la diffusion des produits et services avec des moyens technologiques avancés
- Renforcer la production, la collecte, l'archivage numérique, le développement des bases de données multisources par rapport aux objectifs de ODINAFRICA - II
- Satisfaire la diversité des besoins et leur évolution au regard de la complexité et de la variabilité des usages et de l'environnement marin et côtier

ORGANISATION DU CNDO

- Un coordonnateur sous l'autorité du Recteur,
- Président de l'Université de Lomé
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- En partenariat, des points focaux des institutions publics, privés et ONG produisant et archivant des données

MOYENS DE TRAVAIL

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- Service de préparation des produits
- Service cartographique

RESEAU NATIONAL ET SENSIBILISATION

- Réseau national : 21 sources de données et de l'information
- Sensibilisation nationale : émissions radio et télévision, articles de presse
- Documentaires télévisés
- Activité "Découverte de l'Océan", mars 2003 pour les jeunes des lycées et collèges

Sensibilisation du public lycéen :
Visite du Port Autonome de Lomé

ACTIVITES

- Cours de restitution intra CNDO
- Réunion périodique avec les points focaux
- Cours de formation aux points focaux
- Levé hydrographique
- Numérisation de base de données et de métadonnées
- Relation avec les CNDO du Bénin, du Gabon et de la Côte d'Ivoire

Réunion de coordination avec les points focaux

Atelier de formation des points focaux sur l'élaboration des bases de données

Invitation

Tuesday 31th of May at 16.00 h Gregory Maes will defend his PhD entitled

Evolutionary consequences of a catadromous life-strategy on the genetic structure of European eel (*Anguilla anguilla* L.)

In the Main Auditorium of te Zoological Institure

Naamsestraat 59, 3000 Leuven

LIST OF ABREVIATIONS

α	=	Critical level of significance
AA	=	*Anguilla anguilla*
AAT	=	Aspartate aminotransferase
ADH	=	Alcohol dehydrogenase
AFLP	=	Amplified Fragment Length Polymorphism
AJ	=	*Anguilla japonica*
AM	=	*Anguilla marmorata*
AMOVA	=	Analysis of Molecular Variance
ANOVA	=	Analysis of Variance
AR	=	*Anguilla rostrata*
As	=	Arsenic
ASD	=	Allele Sharing Distance (Bowcock *et al.*, 1994)
BX AA	=	Backcross between F1 hybrid and *Anguilla anguilla*
BX AR	=	Backcross between F1 hybrid and *Anguilla rostrata*
CAGE	=	Cellulose Acetate Gel Electrophoresis
Cd	=	Cadmium
CI	=	Condition Index
C_i	=	Individual concentration of heavy metal i
C_{imax}	=	Maximum concentration of heavy metal i
Cr	=	Chromium
Cu	=	Copper
d^2	=	Squared difference in repeat units
D_{CE}	=	Cavalli-Sforza &Edwards Chord Distance (1967)
DNA	=	Deoxyribose nucleic Acid
DOI	=	Den Oever (glass eel) recruitment Index
DTT	=	Dithiothreitol
EtaS	=	Correlation of genetic identities
F_1	=	First generation hybrid
F_2	=	Second generation hybrid (F_1 x F_1)
f	=	Female
FAC	=	Factorial Analysis of Correspondence
FH	=	Fumarate Hydratase
F_{IS}	=	Inbreeding coefficient
F_{IT}	=	Global inbreeding coefficient
F_{ST}	=	Fixation index
$F_{ST(RB)}$	=	Fixation Index following Raufaste & Bonhomme, 2000
G	=	Glass eel
GF-AAS	=	Graphite Furnace Atomic Absorption Spectrometry
GPI	=	Glucose-6-phosphate isomerase
G_{ST}	=	Coefficient of genetic differentiation
H&W	=	Hardy-Weinberg equilibrium
H_e	=	Expected Heterozygosity
HFC	=	Heterozygosity-Fitness Correlation
Hg	=	Mercury
H_o	=	Observed Heterozygosity
H_S	=	Gene Diversity
HSI	=	Hepatosomatic Index

IBD	=	Isolation-by-Distance
IBT	=	Isolation-by-Time
ICES	=	International Council for the Exploitation of the Sea
ICP-OES	=	Inductive Coupled Plasma-Optical Emission Spectrometry
IDDH	=	L-Iditol dehydrogenase
IDHP	=	Isocitrate dehydrogenase
IIS	=	Identical-in-State
IMBI	=	Individual (multi) Metal Bioaccumulation Index
k	=	Number of simultaneous tests for sequential Bonferroni correction
K	=	Condition Index
K	=	Number of clusters calculated in Structure
K-W	=	Non-parametric Kruskall-Wallis test
L	=	Standard Length
L	=	Liver
LDH	=	Lactate dehydrogenase
M	=	Muscle
m	=	Male
MCMC	=	Markov-Chain Monte-Carlo simulations
MDH	=	Malate dehydrogenase
MDS	=	Multidimensional Scaling Analysis
MEP	=	Malic Enzyme
MLH	=	Multilocus Heterozygosity
MNA	=	Mean Number of Alleles
MPI	=	Mannose-6-phosphate isomerase
m_R	=	Estimator of Admixture proportion using gene frequencies
mtDNA	=	Mitochondrial DNA
m_Y	=	Estimator of Admixture proportion using gene frequencies and coalescent information
MyA	=	Million years ago
N	=	Number of individuals
NAOI	=	North Atlantic Oscillation Index
NAST-CZ	=	North Atlantic Sub-Tropical Convergence Zone
nDNA	=	Nuclear DNA
N_e	=	Effective population size
NEU	=	Northern Europe
ng	=	Nanogram
Ni	=	Nickel
Nm	=	Gene flow
p	=	p-value
$P_{(0.95)}$	=	Level of polymorphism following the 95% criterion
$P_{(0.99)}$	=	Level of polymorphism following the 99% criterion
Pb	=	Lead
PCA	=	Principal Component Analysis
PCB	=	Polychlorinated Biphenyls
PCR	=	Polymerase Chain Reaction
PGDH	=	6-phosphogluconic dehydrogenase
PGM	=	Phosphoglucomutase
q	=	Assignement or Admixture coefficient
R	=	Allelic Richness

r	=	Pearson or Spearman rank correlation coefficient
RAPD	=	Random amplified polymorphic DNA markers
RFLP	=	Restriction Fragment Length Polymorphism
RNA	=	Ribonucleic Acid
rpm	=	Rears per minute
R_{ST}	=	a measure of genetic differentiation analogous to F_{ST}, taking allele size into account
r_{xy}	=	Average relatedness following Queller & Goodnight (1989)
S	=	Silver eel
S.D.	=	Standard deviation
ScnDNA	=	Single-copy nuclear DNA
Se	=	Selenium
SEU	=	Southern Europe
SGE	=	Starch Gel Electrophoresis
SSCP	=	Single-Strand Conformational Polymorphism
SSR	=	Single Sequence Repeat
TG	=	Tris Glycine
TM	=	Tris Malate
TNV	=	Total Number of Vertebrae
VNTR	=	Variable Number of Tandem Repeats
W_B	=	wet body Weight
WEU	=	Western Europe
W_L	=	Liver Weight
Y	=	Yellow eel
Zn	=	Zinc

INTRODUCTION

1. Foreword and aims

The oceans cover more than 70% of the earth's surface and harbour about 10% of the global species diversity. Nevertheless, the marine environment remains much less studied than the continental fauna and flora; most likely the cause can be attributed to methodological constraints (Strathmann, 1993; Largier, 2003). For decades, marine research has amongst others focused on the population dynamics of marine species. Fisheries data are the main source of information on population composition, numbers, stability and fitness of commercial species. From the late 1980s onwards it became clear that the population size of commercial marine species was not inexhaustible, as believed in late 19th century by Thomas Huxley, then president of the Royal Society of London. Many marine fish stocks are fully exploited or even overexploited (Myers & Worm, 2003), prompting for increased accuracy in the estimation of stock structure and size. However, due to the lack of obvious barriers in the marine realm, uncontrolled overfishing has brought many stocks to the brink of extinction (Hauser *et al.*, 2002; Myers & Worm, 2003). To discriminate independently evolving populations, genetic markers have proven to be an invaluable tool (Park & Moran, 1994; Ward, 2000). Additionally genetic information provides indirect estimate of dispersal, population size, demography and stock sustainability (Palumbi, 1994; Waples, 1998; Avise, 2004). Genetic research on marine populations has increased considerably, allowing for better comparisons among species and increasing the knowledge on the interactions between populations and their environment (Beheregaray & Sunnucks, 2001; Nielsen *et al.*, 2001; Planes & Fauvelot, 2002; Arnaud-Haond *et al.*, 2004; Avise, 2004; O'Reilly *et al.*, 2004; Roman & Palumbi, 2004). Studying marine fish requires standardisation of sampling methods in order to increase the signal/noise ratio, which is unfortunately very low in marine

organisms (Waples, 1998). Although the analysis of spawning stocks is the standard, this is not always possible due to the peculiar life history of some species, such as deep-sea species, or species with a remote spawning area (Waples, 1998).

The European eel (*Anguilla anguilla* L.) has attracted the attention of mankind for ages. Aristotle already performed experiments with this species, which he believed to originate by spontaneous generation from mud pools. It is only when Johannes Schmidt (1904) caught 7 mm-long leptocephali larvae of the European eel that research on this species really started (Dekker, 2003). Schmidt subsequently discovered the Sargasso Sea as probable spawning place by following an inverse gradient of leptocephali size (Tesch, 2003). He also suggested the two species concept (*A. anguilla* and *A. rostrata*) in the North Atlantic based on the number of vertebrae. Although intensive research has been conducted for more than a century, the life-history of the eel remains poorly understood. Spawning behavior, larval migration time, sexual determination and differentiation, metamorphoses, directional migration cues and the population genetic structure are still matters of discussion (Arai *et al.*, 2000; Tesch, 2003). Its catadromous life-strategy makes this species completely dependent on natural oceanic conditions and anthropogenic continental influences (Tsukamoto *et al.*, 2002).

The European eel is beyond safe biological limits (Dekker, 2003). Fisheries data indicate that the eel stock is at its historical minimum; only 1% of the 1960 recruitment level is reached at the moment. Several causes have been proposed for the decline ranging from pollution, overfishing, migration barriers, habitat destruction, parasites and diseases to global oceanic and climatic changes (Castonguay *et al.*, 1994; Dekker, 2003; Knights, 2003). Synergy between all these causes seems most likely (Wirth & Bernatchez, 2003). The dramatic decline in other eel species indicates a general trend in commercially fished eel species, urging for immediate decisions on rescue management to avoid complete extinction. Knowledge on the genetic structure of an endangered species enables the use of complementary information for efficient management decisions (Ward, 2000; Hansen *et al.*, 2001). In practice, genetic data can help defining species integrity within the North-Atlantic, identifying the number of genetic stocks within the European eel, spatio-temporal stability of the genetic structure, influences of oceanic conditions on genetic variability, the effect of a population decline on the genetic variability and fitness of eel, and assessing whether such decline has already occurred earlier on, during the Ice Ages.

In this PhD thesis, I study the spatio-temporal genetic structure of the European eel (*A. anguilla* L.), taking into account new knowledge on its biology, with special emphasis on the evolutionary consequences of its catadromous life-strategy. To tackle the problem of temporal

fluctuations, a common feature in marine species, I designed a study that considerably reduced the genetic "noise" and enabled the detection of the biological "signal" within this species. By sampling at various geographical locations for several years and by screening recruiting juveniles and migrating adults with various morphological and genetic markers, many sources of bias were eliminated. The insights should be of special importance to fisheries and conservation management. An additional aim of this work is to integrate oceanic knowledge into genetic studies, and to assess whether subtle genetic structure in the "homogeneous" oceans can be detected with standardized sampling approaches. The evolutionary consequences of overfishing, pollution and strongly fluctuating oceanic conditions can be high, namely by decreasing population sizes, reducing genetic variation, increasing mortality due to genetic load and, if correlated to fitness, putting the whole species in danger of extinction (Thorpe *et al.*, 2000; Hauser *et al.*, 2002; Turner *et al.*, 2002; Hutchinson *et al.*, 2003).

2. The North Atlantic European eel *Anguilla anguilla*

The focal species in this thesis is the European eel. To fully comprehend the influence of its peculiar life-history (catadromy) and huge trans-oceanic migrations on its genetic architecture, I will first provide an overview of its taxonomy, biology, ecology, population dynamics and fisheries status. Then, the origin of the *Anguilla* genus will be presented, followed by a synopsis of the population genetic knowledge within *Anguilla anguilla* and its affinities to its sympatrically reproducing sister species *A. rostrata*.

Taxonomy

The European eel belongs to the family of the Anguillidae. In the genus *Anguilla*, a total of 15 species are recognized (see further). Table 1 depicts the taxonomy of the European eel (*Anguilla anguilla* L.).

Table 1 : Taxonomy of the European eel following Nelson (1994)

Classis	Actinopterygii	**Subordo**	Anguilloidei
Subclassis	Neopterygii	**Familia**	Anguillidae
Divisio	Teleostei	**Genus**	*Anguilla*
Subdivisio	Elopomorpha	**Species**	*Anguilla anguilla* L. (Linneus, 1758)
Ordo	Anguilliformes		

Biology

Eels have a strongly elongated body form and a relatively narrow head, resulting in a more posterior position of the gill rakers than in other bony fishes. There are two obvious phenotypes of the skull structure, namely narrow and broad-headed eels, related to differing feeding habits. Growth rate is higher in broad headed eels and they differ in diet. The lower jaw is more extended than the upper jaw. The unpaired dorsal and anal fins are confluent with the tail fin, which is only visible internally. Ventral fins are lacking and the pelvic girdle is not connected with the skull. The spoon-shaped pectoral fins of the yellow eel become longer and pointed in the silver eel (Tesch, 2003) (Figure 1).

The number of vertebrae is highly polymorphic in anguillids and is often used as determination characteristic between species. For example American eel (*A. rostrata*) and European eel (*A. anguilla*) can be separated on the basis of this feature as the mean values differ strongly (107.2 for American and 114.6 for the European eel). Even across Europe within the European eel, there is some subtle variation on a latitudinal gradient (Harding, 1985). The high number of myomeres, together with the elongated body structure enables a snake-like propulsion (Tesch, 2003).

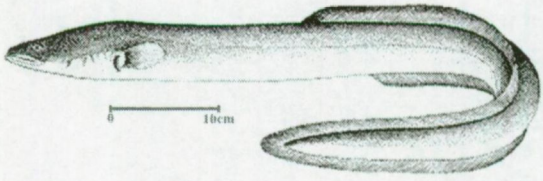


Figure 1 : The morphology of the European eel, *Anguilla anguilla* (L. 1758) (after Bauchot *et al.*, 1987 from Tesch, 2003)

The skin of the eel is very thick, with strongly developed secretory cells for mucus secretion. The rudimentary scales are embedded under the epidermis in the upper layer of the corium. In *Anguilla* spp., they do not develop directly after the larval stage, but appear later. The pigmentation of eel can be used to discriminate developmental stages in juveniles. In the beginning, the planktonic leptocephalus larvae are completely transparent, but slowly develop as well internal as external pigmentation, while transported to Europe by oceanic currents. They do not feed during the early glass eel stages, but start feeding on detritus as soon as pigmentation starts appearing (from stage VB), until they feed on any living material of manageable size when pigmentation progresses (from stage VIA_{III}) (Tesch, 2003). Glass eels migrating along the continental shelf show the same strong negative phototactic behaviour as

at larval stage and will gradually exhibit rheotaxis (swimming against current) and thigmotaxis (searching for mechanical contact). Simultaneously, a pigmented "skull spot" appears, protecting the epiphysis against excessive light, which is coupled with the transition of a pure pelagic to a more benthic lifestyle. From this moment on, glass eel will start to feed actively and will develop additional pigmentation. When juvenile eels are entirely coloured, they reach the yellow eel stage, where pigmentation is entirely dependent on their habitat. Studies have shown that translocated animals are able to adjust their coloration to their new habitat in a few weeks, indicating a strong hormonal process (Tesch, 2003). The yellow eels will feed on living material present in their habitat; monophagy has never been witnessed. There is a difference between narrow headed and broad headed eels in their feeding habit. The first phenotype mainly feeds on larger, more active and difficult to handle preys, such as crabs, crayfish and fish. The narrow headed eels feed mainly on smaller fish and insect larvae from Ephemeroptera and Chironomidae (Tesch, 2003). Competitors for food in freshwater are salmonids, bream (*Abramis brama*) and carp (*Cyprinus carpio*). In front of the coastline, eel can expect high competition with conger eel (*Conger conger*). Predators of eel are birds, such as cormorants and herons and fish (e.g. pike, *Esox lucius*). Cannibalism among eels is also possible. When adult eels are maturing, they change again colour into latero-dorsally grey-blackish and silver-greyish ventrally. At this stage they are named silver eels.

Geographical distribution

The spawning region of the European eel ranges from 23°N to 29.5 °N and from 48°W to 74°W (known as the Sargasso Sea). While overlapping extensively with that of the American eel, spawning is somewhat more southwards (Figure 2). The Sargasso Sea is situated within the North-Atlantic Sub-tropical Convergence zone (NAST-CZ) (Diekmann & Piatkowski, 2002). Leptocephali are found there from mid February to June for the European eel and from mid February to April for the American eel (McCleave, 1993). However, calculations of the age of leptocephali have indicated a second "late" spawning period in European eel from August to November (Desaunay & Guérault, 1997). The larvae of the European eel follow the Gulf Stream, the North-Atlantic Drift and additional branches thereof towards Europe by passive drift and active swimming (Lecomte-Finiger, 1994; Desaunay & Guérault, 1997; Arai *et al.*, 2000, Tesch, 2003). Larvae have been collected in the upper water column layers (60-160 m depth), where they perform diurnal migrations due to negative phototactic behaviour.

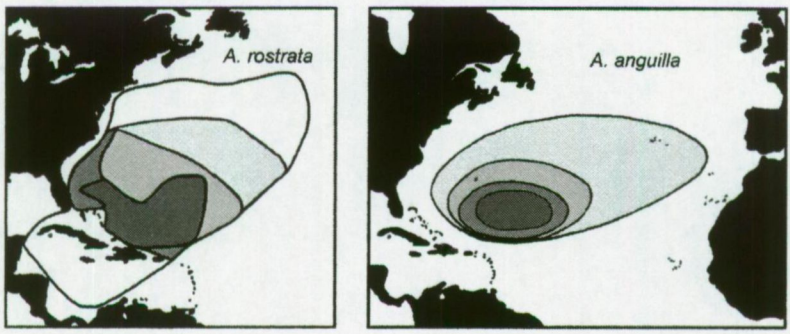


Figure 2 : The distribution of *Anguilla rostrata* (left) and *A. anguilla* (right) in the Atlantic Ocean. The various grey colours represent different length classes (10 mm (dark); 20 mm, 30 mm, 40 mm, 70 mm (white) (Miller, 2003)

The continental shelf represents the division between the leptocephalus stage and the glass eel stage; no larvae are found eastward of the shelf. The most northward location where eel has been found is Cape North (Norway); the Canary Islands constitute the southern boundary of their distribution area (Figure 3). Eastwards and westwards, their distribution is delineated by the Black Sea and Iceland. Due to stocking, eels are found throughout the Danube and Wolga basin (Tesch, 2003).

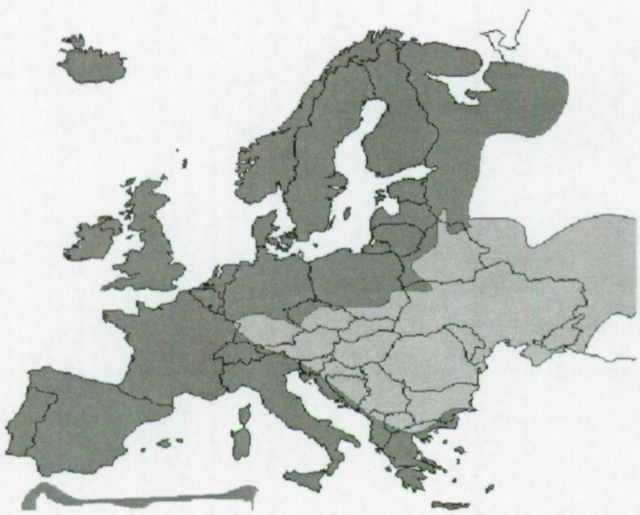


Figure 3 : The continental distribution of *Anguilla anguilla*. The dark grey area is the natural distribution area; the light grey represents stocked populations (adapted from Lelek, 1987).

Life cycle

The catadromous eel reproduces semelparously; the life cycle is composed of an oceanic and continental phase. During their entire life they undergo four complete or partial metamorphoses between the egg, larval, glass eel, yellow eel and silver eel stages (Figure 4). Their life cycle is a prime example of a "migration loop" as described by Tsukamoto *et al.* (2002), where the completion of all developmental stages is highly adapted to a gyral system guiding larvae from the spawning place and adults from their highly dispersed feeding regions. Understanding the catadromous life-strategy is crucial to assess the multiple endogenous and exogenous influences on eel, which shape its population structure. Despite mostly being regarded as a freshwater species, the European (and American) eel represent fully marine species, under the influence of oceanic and climatical forces determining their survival and population dynamics.

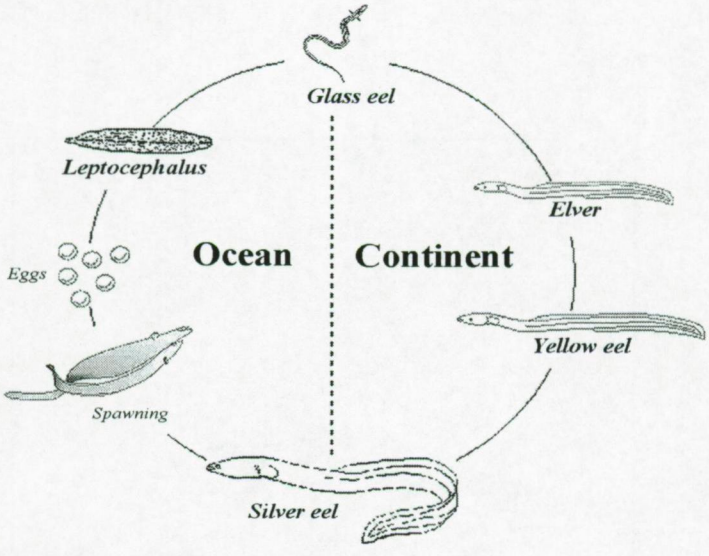


Figure 4 : Summary of the life-cycle of *Anguilla spp*, showing the major stages.

The Oceanic phase

European eel reproduces during a rather protracted spawning season generally around the months February-April (McCleave, 1993), but can also spawn later on in summer (Desaunay & Guérault, 1997). Leptocephali from American and European eel hatch in the Sargasso Sea, where they survive the first days without food uptake, but from energy stores in an oil droplet (yolk sack). They start to feed on particulate organic material (Mochioka, 2003). Despite their

adaptation to an oligotrophic environment, their survival is entirely dependent on the phytoplankton bloom in the Sargasso Sea in spring. The timing of spawning relative to the phytoplankton bloom has therefore important consequences on the annual reproductive success of adults, resulting in a protracted spawning period to maximize larval survival (Tesch, 2003). The larvae are then transported to the European continent, with a migration depth of 60-160 m. Because of their planktonic migration, recruitment is dependent upon oceanic currents (Figure 5). The transoceanic migration phase of leptocephali remains a matter of discussion. Considering purely passive drift along North-Atlantic currents, larvae should take two to three years to reach the European continent (Tesch, 2003). On the other side, based on controversial daily otolith ring increment calculations, larval migration is now believed to last for only seven to nine months (Desaunay & Guérault, 1997; Arai *et al.*, 2000). In any case an active swimming component must be present to migrate against or pass strong currents (Tesch, 2003). Once arrived at the continental shelf, they metamorphose into glass eels. Their bodies will shorten, become more cylindrical and loose much water, while gradually pigmenting.

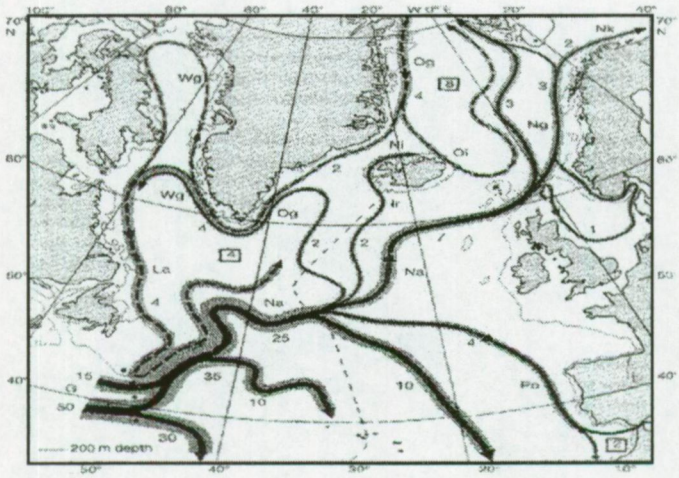


Figure 5 : Currents in the North Atlantic Ocean above 2000 m depth bringing leptocephali larvae to the European continent. G: Gulf Stream; Ir: Irminger Current; La: Labrador Current; Na: North Atlantic Current; Ng: Norwegian Current; Nk: North Cape Current; Ni: North Iceland Current; Og: East Greenland Current; Oi: East Iceland Current; Po: Portugal Current; Sb: Spitzbergen Current; Wg: West Greenland Current. (Tesch, 2003)

The continental phase

Glass eels continue their migration along the continental shelf in the direction of estuaria, where they reach the French and Spanish coasts around September and the English Channel around January-February. From September till January, they advance gradually from Northern Scotland to the Dutch coasts and the Baltic Sea, where they arrive around February/March (Figure 6). Glass eels reach Gibraltar even earlier than the Gulf of Biscay. They use tidal transport and their own swimming capacity to ascent rivers. Temperature and salinity are crucial factors influencing the migration behaviour of glass eels, as they only ascent rivers at a temperature between 9-12 °C (Dekker, 2003).

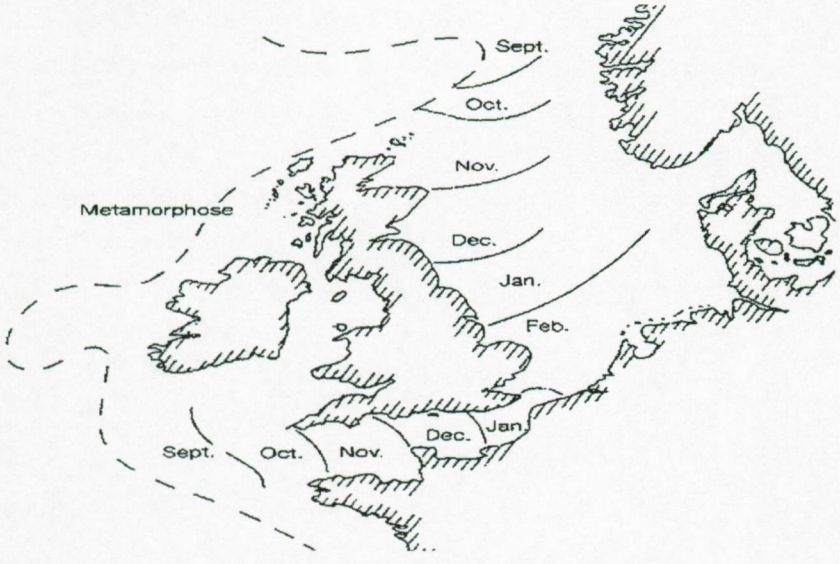


Figure 6 : Migration course of glass eels over the continental shelf (Tesch, 2003).

From a length of 20 cm, subadults are named yellow eels, which will grow for several years in the rivers. A part of the population may remain completely or partially (estuarine) marine (Tsukamoto *et al.,* 1998). There is a strong sexual dimorphism with respect to growth rate, males growing much slower than females (Tesch, 2003) (Figure 7). The geographical distribution of the sexes is biased towards more females northwards/upstream and more males southwards/in estuaries. The sexual determination is still unresolved till today, given that environmental factors such as temperature, salinity, population density and food availability may play a role (Beullens *et al.*, 1997; Tesch, 2003).

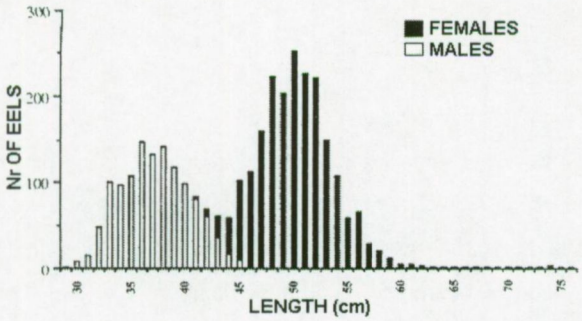


Figure 7 : Example of the length distribution of European eel by sex in the Burrishoole system, Western Ireland (Poole & Reynolds, 1996).

After the growing period in the rivers, partially mature silver eels start their migration to the Sargasso Sea. The start of migration depends on the location; in The Netherlands they start in August, while in Ireland and the Baltic Sea they leave in October. Together with the silvering of the skin, other morphological adaptations emerge, such as eye enlargement, digestive tract reduction and osmoregulatory changes (Aoyama & Miller, 2003). Age and length at the silver eel stage differs as well in time as space, as a consequence of climatic differences, food availability, trophic level and fisheries pressure (Poole & Reynolds, 1996). Poor climatic conditions, strong oligotrophic systems, high predation pressure and selective fisheries for fast growing (sub)adults can significantly reduce size at maturity.

Population dynamics, fisheries status and aquaculture

The European eel is outside safe biological limits. Fisheries data indicate a historically low recruitment level. The decline in eel most likely started around 1950, the recruitment crashed to only 10% of this level around 1980 (Dekker, 2003). In 2004, glass eel recruitment averaged 1% of the 1960 level. The same trend is visible in other eel species worldwide (Figure 8). There have been several hypotheses concerning the causes of the eel stock decline during the second half of the century. On the one hand, there are several anthropogenic factors influencing eel reproductive success, such as migration barriers (dams and hydroelectric power plants), overfishing, pollution (PCBs and heavy metals), habitat destruction, diseases (EVEX virus) and parasites (the swimbladder nematode *Anguillicola crassus*) (Lefevre *et al.*, 2002; Robinet & Feunteun, 2002).

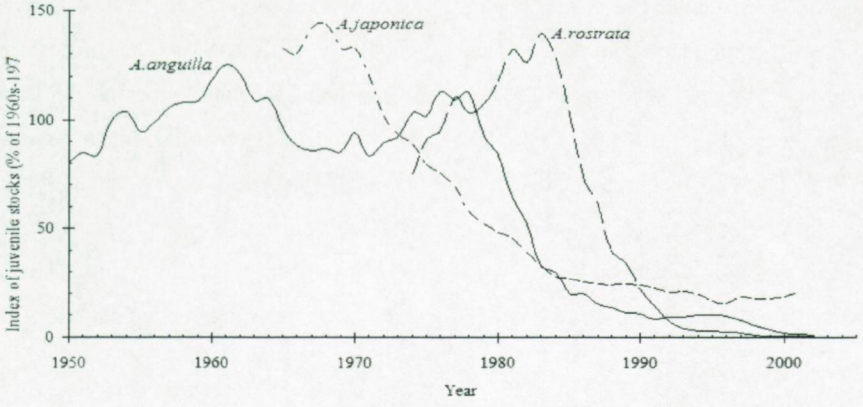


Figure 8 : Time trends in juvenile abundance of the major eel stocks of the world. For *Anguilla anguilla*, the almost-continental wide average trend of the four longest data series is shown; for *A. rostrata*, data represent recruitment to Lake Ontario; for *A. japonica*, data represent landings of glass eel in Japan (Dekker *et al.*, 2003).

On the other hand, there is strong evidence of a correlation between climatic and oceanic events and recruitment success (Castonguay *et al.,* 1994; Dekker, 1998; Knights, 2003). The Den Oever recruitment Index (DOI) monitoring glass eel arrival in The Netherlands, was strongly negatively correlated with changes in the North Atlantic Oscillation winter Index (NAOI) over the last decennia (Figure 9). It is thus likely that a synergy of negative effects has caused the decline of eel (Wirth & Bernatchez, 2003).

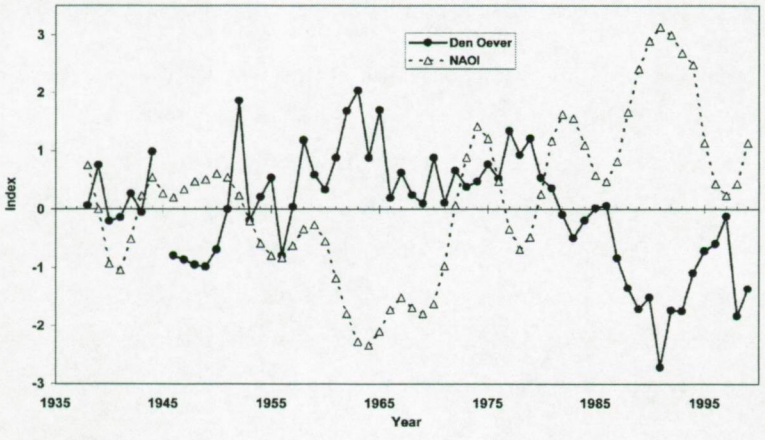


Figure 9 : DOI (5 year average, dots) and NAOI (5 year Fast Fourier Transform average, open triangles) over 1938–1999 (replotted from Westerberg, in ICES, 2001; after Knights, 2003).

Dekker (2004) estimated the census population size of the European eel from fishery data by life stage. As well glass eels as silver eels are heavily exploited. England, France, Spain, Portugal and Morocco are fishing glass eels for consumption, while more northerly regions use glass eels for restocking their depleted rivers (Dekker, 2003). An average of 2.1×10^{12} glass eels recruit annually, but only about 14% is estimated to escape the fishery, freely migrate upstream and grow to yellow eels (reduced to 5% when considering natural mortality). A roughly estimated 9×10^{6} spawners escape from the continent to safeguard the next generation, corresponding to a 99.5% accumulated life-span fishing mortality (Figure 10).

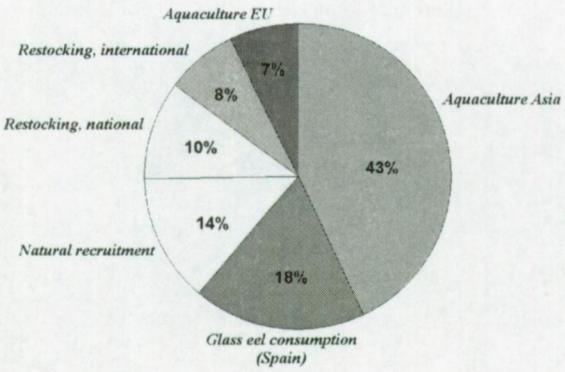


Figure 10 : Destination of caught glass eels of *Anguilla anguilla*. Only 14% swim upstream (Dekker, 2003).

Yellow and silver eels are mainly fished in central-Europe. The total catch of yellow and silver eels is estimated at 20,000 tons per year (Dekker, 2003). The present exploitation pattern reduces the escapement of spawning individuals to a low 2.5 – 12.5% of the unexploited level (calculated resident biomass), while 30% would represent a minimum. A restriction in fisheries pressure is thus urgently needed. A precautionary approach has been proposed to protect the European eel stock at a regional, national and international level (Starkie, 2003). Integrating data and knowledge of the marine part of its life-cycle is a next step in the direction of a global management strategy.

3. The North Atlantic Ocean: Oceanography and climatic influences

Highly vagile marine species are usually left to the mercy of oceanographic and climatic forces. The European eel's adult reproductive success, larval dispersal potential, post-hatching survival and early life stage selection depend on the ruling conditions in the North-Atlantic Ocean. The Atlantic is the world's second largest ocean and several topographic features distinguish it from other oceans. First, the Atlantic Ocean extends into the Arctic and Antarctic, giving it a total meridional extent of over 21,000 km. Second; it has the largest number of adjacent seas, including the Mediterranean Sea, influencing the characteristics of its waters. Third, the Atlantic Ocean is divided rather equally into a series of eastern and western basins by the Mid-Atlantic ridge, which in many parts rises to less than 1000 m depth, reaches the 2000-m depth contour nearly everywhere, and consequently has a strong impact on the circulation of the deeper layers (Ottersen *et al.,* 2004). The upper water circulation of the Atlantic Ocean consists in its gross features of two great anticyclonic circulations or gyres, a counterclockwise one in the South Atlantic and a clockwise one in the North Atlantic (Figure 11). The two gyres are driven separately, each by the trade winds in its own hemisphere, and they are separated over part of the equatorial zone by the eastward flowing Counter Current (Ottersen *et al.,* 2004). The clockwise gyre starts with the North Equatorial Current, flowing towards the northwest as the Antilles Current, escaping between Florida and Cuba into the North Atlantic as the Florida Current and after joining up with the Antilles Current, the joint current breaks away from the North American shore, now called the Gulf Stream.

The Gulf Stream flows northeast to the Grand Banks of Newfoundland at about 40°N and 50°W, after which it continues east and north as the North Atlantic Current. It is driven by the Trade Winds, which cause sea levels to be higher in the western part of the Atlantic basin and generate an area of high water pressure centered on the Sargasso Sea, comparable to the anticyclonic cells of the atmosphere. However, the Coriolis force, caused by the earth's rotation, deflects the geostrophic flow to the right in the Northern Hemisphere. Ocean currents, therefore, describe a clockwise gyre in the tropical Atlantic, and as the higher sea level is found in the west, the current that flows along the American coast is narrow and fast.

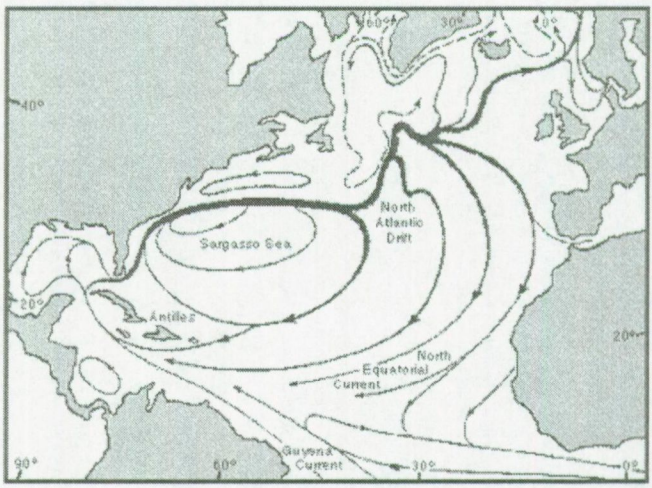


Figure 11: The main currents of the North-Atlantic gyral system (source: http://www.howe.k12.ok.us/~jimaskew/bio/bbiome.htm).

A number of physical processes may influence marine life at the individual, population, and community level. Processes that influence the reproductive habitat of many species of fish were grouped into three main classes by Agostini & Bakun (2002): (i) *Enrichment processes*: upwelling, mixing, cyclonic wind stress curl and cyclonic eddy formation; (ii) *Concentration processes*: convergent frontal formation, anticyclonic wind stress curl and lack of dispersion by turbulent mixing processes; (iii) *Retention processes*: lack of offshore transport in near-surface and superficial layers, geostrophic current (intermediate layers), and offshore eddy-like features (filaments) on the meso-scale. Simplified, e*nrichment processes* make more nutrients available to biological productivity, c*oncentration processes* enhance food availability for a predator through increasing the concentration of food particles, and *retention processes* contribute towards keeping individual members of a population in the appropriate place during the various parts of the life cycle. Oceans are highly influenced by climatic fluctuations, as currents are mainly wind-driven. One major atmospheric phenomenon, amongst others, influencing the North-Atlantic is the North Atlantic Oscillation. The North Atlantic Oscillation (NAO) is a north-south alternation in atmospheric mass between the subtropical atmospheric high-pressure center over the Azores and the atmospheric subpolar low-pressure center over Iceland. It measures the strength of the westerly winds blowing across the North Atlantic Ocean between 40°N and 60°N. Variability in the direction and magnitude of the westerlies is responsible for fluctuations in wintertime temperatures and the balance of precipitation and evaporation across the Atlantic and the adjoining landmasses

(Stenseth *et al.*, 2002). During positive phases of the NAO, the westerly winds are strengthened and moved northward, causing increased precipitation and temperatures over northern Europe and the southeastern United States, and dry anomalies in the Mediterranean region. Roughly opposite conditions occur during the negative-index phase (Stenseth *et al.*, 2002).

The ecological effects of the NAO can be classified according to three major types: direct, indirect and integrated effects (Ottersen *et al.*, 2004). The classification of response to change in climate as either direct or indirect makes sense also at the community level. Changes in temperature that influence, for example, growth and recruitment, may lead to a direct response on fish species in the community, with the abundances of individual species changing in a predictable manner according to each species' optimum for growth and reproduction. On the other hand, climate shifts may lead to, for example, variability in oceanic circulation patterns and temperature-induced changes in prey abundance. Fish communities may then act indirectly to a change in climate by way of responding to changes in local abiotic or biotic conditions. Related to the issue of direct and indirect climate effects is the question of time lags in ecological responses to climatic variation. Ottersen *et al.* (2004) showed how some of the effects of the NAO may influence a biological population over a number of years. The increase in survival through the vulnerable early stages of Barents Sea cod during warm, high NAO years historically results in stronger year classes, at later catchable stages (Stenseth *et al.*, 2002). When such year classes mature, the number of spawners as well as their individual size may be increased, compared to low NAO year classes. This enhances the potential for high recruitment to the next generation. On the other hand, the year-class strength of cod in the North and Irish Seas is inversely related to a positive NAO phase and high temperature. This is possibly a result of limitation in energy resources necessary to achieve higher metabolic rates during warm years (Planque & Fox, 1998). In both cases, the effects of the NAO are perceived in the fisheries with a lag of several years.

Climatic fluctuations may affect the relative timing of food requirement and availability. Survival of a predator depends on its ability to encounter and eat a sufficient quantity of suitable prey in order to avoid starvation and to grow. Differences in the temporal and spatial match between predator and prey may thus generate variability in predator survival rates, including interannual variability. This 'match–mismatch' hypothesis was first presented by Hjort (1914) and updated by Cushing (1990) some 30 years ago. Match–mismatch type mechanisms have been reported for different regions and at different trophic levels, often in

its original context (relating cod larvae to their zooplankton nauplii prey), but also for other species, such as herring in the St Lawrence estuary (eg Fortier & Gagné, 1990). The timing of the spring bloom depends on the supply of light to the phytoplankton in spring, which in turn critically depends on the mixing characteristics of the upper water masses. This bloom is based on 'new' nutrients admixed to the upper layers during winter. Differences in the timing of spring blooms affect the match between phytoplankton and zooplankton maxima. To maximize grazing and minimize sedimentation ('match'), phytoplankton blooms should not begin too early and they should be of long duration so that the probability of zooplankton stocks encountering phytoplankton blooms is maximized.

4. Evolution in marine fishes: biological and genetic consequences of catadromy

Life history traits of marine species occupying a large geographic area are usually entirely under the influence of oceanographic and climatic conditions, dictating adult reproductive success, larval dispersal potential, post-hatching survival and early life stage selection (Palumbi, 1994; Waples, 1998). The high fecundity of many marine fishes ($10^4 - 10^7$ eggs per spawning period) together with external fertilization increases the chance for dispersal and mortality, dependent on the time till larval settlement in the nursery habitat and the oceanic conditions (Cowen *et al.,* 2000; Largier, 2003). Such a life history has important population genetic consequences: a high genetic variability (Palumbi *et al.,* 1994; Ward *et al.,* 1994; DeWoody & Avise, 2000), a significantly larger amount of gene flow than in freshwater fishes, a reduced spatial genetic differentiation (Ward *et al.,* 1994; De Innocentiis *et al.,* 2001; O'Reilly *et al.,* 2004; Pampoulie *et al.,* 2004), and supposedly large effective population sizes (Avise, 2004; but see Hauser *et al.,* 2002). However, marine species do not use their full dispersal capacity for several reasons. Homing and assortative mating behaviour, frontal or other "invisible" barriers and larval retention may all significantly reduce gene flow (Sinclair, 1988; Avise, 2004). Despite the usually high number of spawners, marine organisms are susceptible to a very high reproductive variance, inducing large or small-scale genetic heterogeneity among and within local populations (Hedgecock, 1994; Li & Hedgecock, 1998; David *et al.,* 1997; Johnson & Wernham, 1999; Planes & Lenfant, 2002; Flowers et al, 2002). The study of temporal variation in marine organisms is of crucial importance to discriminate between temporal stochastic genetic heterogeneity between successive cohorts (Johnson & Black, 1982; Hedgecock, 1994; Planes & Lenfant, 2002) and the existence of temporally distinct reproductive units within and between spawning periods (Hendry & Day, 2005).

Temporal genetic heterogeneity can be explained by two main hypotheses, which are not mutually exclusive. The first hypothesis attributes genetic variation between cohorts at a microgeographical scale to temporal variation in the genetic composition of recruits. The unpredictability of the marine environment can lead to a differential contribution of parents and subsequent temporal variation in the genetic composition of the recruits (Hedgecock, 1994; David *et al.*, 1997; Li & Hedgecock, 1998; Johnson & Wernham, 1999; Planes & Lenfant, 2002). Under the hypothesis of "sweepstakes reproductive success" (Hedgecock 1994), chance events determine which adults are successful in each spawning event. Hedgecock (1994) attributed the variation in reproductive success of adults to spatio-temporal variation in oceanographic conditions, occurring within and among seasons. Fluctuations in recruitment success are highly dependent on the match with food availability at the spawning place (match-mismatch hypothesis; Cushing 1990, 1996) and the degree of stability of retention zones (member-vagrant hypothesis; Sinclair, 1988). The difficulty to assess the exact timing of ideal environmental conditions at the spawning place and the high larval mortality rate in type III survival organisms (producing large numbers of offspring, few of which survive), may force marine species to distribute their reproductive effort among several spawning events within a reproductive season, a strategy similar to bet-hedging (Flowers *et al.,* 2002). The genetic consequences of such high variance in reproductive success is the induction of an unpatterned genetic constitution of recruits (genetic patchiness), that can counteract forces restricting dispersal in space and time. Importantly, genetic differentiation between populations at a local scale may exceed differentiation at larger scales. Allele frequencies at selected markers can also be influenced by pre- and post-settlement selection, often inducing clinal variation across environmental gradients (Hedgecock, 1994; Johnson & Wernham, 1999).

A second set of hypotheses ascribes temporal differentiation to the sampling of biologically and genetically different source populations. The "member-vagrant" larval retention hypothesis (Sinclair, 1988) suggests that mature adults use spawning sites with high ecological value to maximize the survival of their larvae, which actively remain in such area for a few weeks. Vagrants are believed to experience high mortality, although they might be important for the stability of the entire population during bad recruitment years (McQuinn, 1997). Such retention zones can be spatially separated or can be used by temporally separated spawning cohorts, with a higher chance of mixing between populations in the latter case (Stepien *et al.*, 1999). The extent of overlap between spawning groups will determine the level of inter- or intra- annual differentiation between cohorts. If some populations overlap

strongly, a temporal Wahlund effect will be observed within samples (Wahlund, 1928), whereas when overlap is low or absent a pattern of Isolation-by-Time (IBT) can be detected between consecutive cohorts (Hendry & Day, 2005). The stability of this pattern is dependent on the heritability of reproductive times in early and late spawners at a specific spawning site. Adults with different and specific spawning time will generate offspring of an intermediate reproductive value. The lower the heritability of spawning time, the higher the temporal gene flow through dispersal will be (Hendry & Day, 2005). Isolation-by-Time has never been detected or tested in marine organisms, most likely due to the subtle spatio-temporal variation in such organisms and the less defined or stable spawning regions (Waples, 1998). Nevertheless, several studies investigating temporally separated marine populations had the chance to test for IBT, such as in cod, red drum and plaice, where successive larval or adults samples were collected in putative retention zones (Ruzzante *et al.*, 1996, 1999; Beacham *et al.*, 2002; Hoarau *et al.*, 2002).

Fish migration is typically defined as a regular habitat transition between the spawning area and growth habitat of a species. A conceptual model that superimposes both the life cycle of migratory fishes and the circular route of migration connecting the spawning area and growth habitat has been defined as a "migration loop" (Tsukamoto *et al.*, 2002). In principle, each fish species has a migration loop peculiar to its life history and geographic distribution. Therefore, the differentiation of a new migration loop would have the potential to cause reproductive isolation and thus speciation. This concept is useful for understanding the evolutionary processes of fish migration as well as the present-day migratory behavior and life cycles of fishes. Diadromous migrations have been observed in several taxa of fishes, suggesting that diadromy originated independently throughout evolution (McDowall, 1992, 1993). Therefore, each migratory fish species might have experienced different evolutionary selection pressures that resulted in variations in its life history.

Diadromy is a migratory strategy in fishes that involves a regular migration pattern between fresh and salt water (McDowall, 1992). It is a relatively rare behavioral trait, occurring in perhaps 250 out of the circa 25,000 known fish species (McDowall, 1993; Nelson, 1994). Diadromous fishes undertake two major habitat shifts in every generation: a migration from fresh water to the ocean, and another migration in the opposite direction. Some diadromous fish are semelparous (one migration in each direction in an individual lifetime), others are iteroparous (two to several migration cycles). Anadromy, catadromy, and amphidromy are all variants of diadromy. Anadromy refers to the migration patterns of fish, such as salmon, that live in the ocean but return to fresh water to spawn. Catadromy refers to

the migration patterns of fish, such as the European eel, that live in fresh water but return to the sea to spawn. In amphidromy, such as in gobies, the migrations are not directly tied to spawning, but to some other activity, such as feeding. The evolution of such life-strategies is triggered by several abiotic and biotic factors, such as glaciations, continental drift, habitat suitability, food availability and selection. For example, the ancestral eel might have been a tropical marine species with a migration loop extending to coastal waters. Incidentally they visited estuaries and eventually obtained a reproductive advantage because of higher food availability in estuaries and then in fresh water. Thus the ancestor of anguillid eels probably developed an adaptive behavior of regularly migrating upstream as a result of a cline in food abundance between the ocean and fresh water in the tropics. For this process to occur, there must have first been a euryhaline marine species and then an oceanic amphidromous species before the appearance of catadromous eels (Tsukamoto *et al.*, 2002) (Figure 12). A similar scenario can be put forward in anadromous fishes, such as in salmonids.

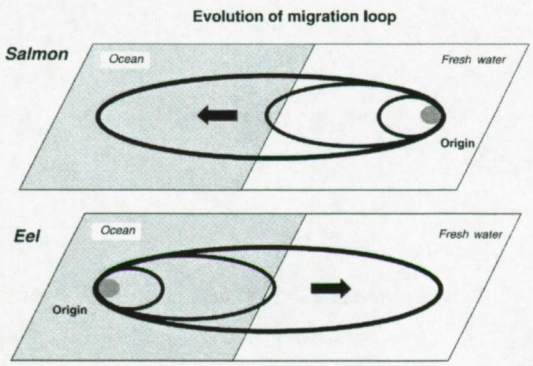


Figure 12 : A diagram representing changing migration loops that result in the evolution of the diadromous life histories of salmon (top) and eel (bottom) (Tsukamoto *et al.*, 2002).

The evolutionary consequences of a catadromous life-strategy in fishes is poorly known, mainly because of a paucity of information on the genetic structuring in this small group of fishes. Very little is known of the biology and ecology of species with a catadromous life-strategy in general (McDowall, 1992). Catadromy has important repercussions for the formation of spatial genetic heterogeneity, because the potential for gene flow among geographical locations is greater than that of purely freshwater fishes. The few studies on genetic structuring in catadromous fishes have shown that the extent of differentiation among populations varies and is closely linked to life-history attributes; in particular, the location of spawning site and the subsequent dispersal of larvae. In barramundi (*Lates calcarifer*) and

Australian bass (*Macquaria novemaculeata*), which spawn at the mouths of coastal drainages, disjunct riverine populations are genetically heterogeneous across Australia (Chenoweth *et al.*, 1998, Jerry & Baverstock, 1998). The restricted genetic differentiation in these two examples demonstrates the need to gather additional information from other catadromous fishes in an effort to understand more on how this particular life-strategy influences spatial genetic population structuring.

5. Heterozygosity-Fitness-Correlations (HFC)

The genetic diversity characteristic of a species is essential to its long term survival for several reasons: Heterozygosity is positively related to fitness, the rate of evolutionary change able to occur in a group of organisms is dependent on the amount of genetic variation present in the gene pool and the global pool of genetic information represents the 'blueprint' for all life (Frankham, 2002). Organisms exhibiting low levels of genomic variability may suffer from a reduced fitness, through the negative effect of inbreeding depression and genetic load of lethal allelic variants. Inbreeding depression can be seen by decreased fertility and fecundity, increased offspring mortality, fluctuating asymmetries and morphological variation and disease susceptibility.

Heterozygosity-fitness correlations (HFC), the correlation between heterozygosity observed at marker loci and fitness-related traits such as growth, survival, fecundity or developmental stability, have been under scrutiny for decades in populations of a wide variety of species. Positive HFC have been reported in organisms as diverse as plants (Schaal & Levin, 1976; Ledig *et al.*, 1983), marine bivalves (Zouros *et al.*, 1980; Koehn & Gaffney, 1984), crustaceans (Bierne *et al.*, 2000), amphibians (Pierce & Mitton, 1982), salmonids (Leary *et al.*, 1984; Danzmann *et al.*, 1987; Thelen & Allendorf, 2001) and mammals (Slate & Pemberton, 2002; Hildner *et al.*, 2003). Nevertheless, null results are likely to be under-represented in literature (Hansson & Westerberg, 2002), although inconsistent and negative correlations have also been documented. In blue mussel *Mytilus edulis*, an initially observed superior viability of heterozygotes was insignificant in a posterior experiment (Gaffney, 1990). In the European oyster *Ostrea edulis*, despite observing a positive relationship between heterozygosity and growth, Alvarez *et al.* (1989) found a negative correlation between allozyme heterozygosity and viability. In the surf clam *Spisula ovalis*, heterozygosity was positively correlated with viability in the 1993-1994 period but not in the 1994-1995 interval and, also depended on the site considered (David & Jarne, 1997).

The correlation between genetic variability and fitness components as reflected by molecular marker heterozygosity in natural populations usually accounts for only a small percentage (1-5%) of the observed phenotypic variance (David, 1998). The first HFCs were observed in studies using allozyme markers, which led to the hypothesis of direct overdominance, where the correlation is due to a direct heterozygous genotype advantage at the studied allozyme loci compared to the corresponding homozygous genotypes. It has been proposed that heterozygotes at allozyme loci might have an intrinsically higher fitness than homozygotes, which would be related to lower energy consumption and greater metabolic efficiency (Mitton, 1993; David, 1998). The observation of positive HFC correlations with putative neutral DNA markers indicates however that at least some correlations are not due to the direct effect of the marker genes but to the genetic association between the neutral markers and fitness genes, resulting in associative overdominance (David, 1998). When marker loci are not directly responsible, the observed lower fitness of homozygotes can be either due to the effect of linkage disequilibrium restricted to a narrow chromosomal section affecting closely-linked fitness loci ("local effect") or due to partial inbreeding caused by non-random association of diploid genotypes in zygotes ("general effect") (David *et al.,* 1995).

Few studies correlating heterozygosity and growth have been carried out in fish, mainly due to the difficulty to carry out rearing experiments, especially for marine species. In salmonids, Danzmann *et al.* (1987) found a positive correlation between allozyme heterozygosity and growth (length or weight up to 6 months) in cultured rainbow trout (*Oncorhynchus mykiss*), suggesting that heterozygotes have enhanced growth rates compared to more homozygous individuals. Nevertheless, salmonids seem to produce different HFCs at different life stages, and negative correlations were found between allozyme variation and length at one year or length and weight at maturation (Ferguson, 1990, 1992). Pogson & Fevolden (1998) examined the relationships between growth and the degree of individual heterozygosity at ten nuclear RFLP loci in two natural populations of Atlantic cod (*Gadus morhua*), using a rough measure of growth (size at age). A significant positive correlation was found in one population, supporting the hypothesis that neutral DNA markers can detect HFC.

Evidence from animal and plant populations indicates that allozymatic polymorphism and heterozygosity might also be linked to environmental heterogeneity and stress (Stanton *et al.*, 2000; Nevo, 2001). Understanding the effects of pollutants on the genome is of crucial importance to preserve the evolutionary potential of endangered natural populations, as a high genetic diversity provides a population the potential to adapt to selective forces (Gillespie & Guttman, 1989). Under natural conditions (e.g. absence of anthropogenic influences), allelic

frequencies within a population fluctuate with time according to stochastic processes (drift), migration and/or environmental selection pressures (such as climate or habitat changes). Severe perturbations on a short temporal scale, such as man-induced pollution and harvesting, may lower both the condition of the population and its genetic variability, reducing the viability (fitness) of natural populations. Hence the susceptibility to additional environmental stress increases, weakening the survival of the species (Thorpe *et al.*, 1981; Leary *et al.*, 1987; Stanton *et al.*, 2000). The importance of genetic variation to survive anthropogenic environmental changes relates to factors such as resistance to heat stress (*Fundulus heteroclitus*; Powers *et al.*, 1991), oil pollution (*Mytilus edulis*; Fevolden & Garner, 1986) and radiation (*Campostoma anomalum*; Gillespie & Guttman, 1989).

The impact of pollutants or toxicants, such as heavy metals, pesticides or industrial waste, on the genetic diversity and structure of natural populations can be important and may be reflected by a reduced genetic variability in polluted populations, genotype-specific survivorship and subsequent shifts in the distribution of tolerant genotypes without net loss of diversity, or significant correlations between the concentration of pollutants and allele frequencies (Hvilsom, 1983; Fevolden & Garner; 1986; Klerks & Weis, 1987; Patarnello & Battaglia., 1992; Posthuma & Van Straalen, 1993). Most of these studies focused on well-defined populations, with low dispersal capability and reproducing locally. The question remains whether the effect of pollutants can also be measured on physical condition and genetic variability in highly vagile species. Organisms with a catadromous life history (i.e. spawning at sea, feeding in rivers and lakes) are special cases. They are expected to reflect the impact of local pollutants faithfully, as somatic and population genetic comparisons can be made after dispersal without worrying about different genetic background, parental influence or larval pollution load. Species with a presumably high effective population size (which are mostly marine) generally exhibit high levels of heterozygosity and are expected to be more resistant to pollution. Recent data reported very low N_e/N ratios in such organisms due to strong variance in reproductive success.

6. Molecular markers in population biology and analytical techniques

The choice of molecular markers in population biology is highly dependent on the organism of study, the availability of tissue, the availability of molecular markers, the sensitivity required and the hierarchical level of the study (species, population, individuals) (Park & Moran, 1994). Figure 1.13 shows the level of variability and applicability of different genetic

markers (Féral, 2002). In this study, we chose to combine three markers used in eel genetic research and compare the signal detected by them: meristic markers (number of vertebrae; Schmidt, 1922), allozymatic markers (Koehn & Williams, 1978) and microsatellite markers (Daemen *et al.,* 1997). Meristic traits are the basis of the North-Atlantic eel discrimination, while allozymes suggest a weak North-South clinal or habitat selective differentiation. Microsatellites are believed to have the highest discrimination potential to detect subtle structure in populations, and they may be the only marker with potential to unravel the genetic structure of eel. Because of its marine life history, we anticipated a high genetic variability, low level of differentiation and a low signal/noise ratio (Waples, 1998) in eel. We therefore chose to screen many allozymatic and microsatellite markers to increase statistical power (see also Figure 13). We did not choose to screen mtDNA for several reasons. First, the analysis of multiple independent nuclear loci has an increased power to reveal structure in lowly differentiated species, as they each represent an independent signal of differentiation (Sunnucks, 2000; Hansen *et al.,* 2001). Second, due to its non-Mendelian mode of inheritance, the mtDNA molecule must be considered a single locus in genetic investigations (Avise, 2004). Third, many new data analysis techniques to detect subtle genetic differentiation have been developed for microsatellite DNA, because of their high variability and codominant inheritance (Hansen *et al.,* 2001; Pearse & Crandall, 2004). Finally, because mtDNA is maternally inherited, the phylogenies and population structures derived from mtDNA data may not reflect those of the nuclear genome due to gender-biased migration or introgressive hybridization (Liu & Cordes, 2004). Unfortunately, mitochondrial and microsatellites DNA markers are both subject to problems, such as back mutation (sites that have already undergone substitution return to their original state, also called homoplasy), parallel substitution (mutations occurring at the same site in independent lineages), and rate heterogeneity or mutational hot spots (large differences in the rate at which some sites undergo mutation when compared to other sites in the same region) (Liu & Cordes, 2004). There are several methods to detect and correct for such bias, such as the sequencing of alleles, although estimates of genetic differentiation proved to be robust to a certain degree of homoplasy (Balloux & Lugon-Moulin, 2002; Estoup *et al.*, 2002). New methods, however, are expected to minimize such bias. For instance, the comparison of genomic DNA sequences in different individuals reveals some positions at which two or more bases can occur. These single nucleotide polymorphisms (SNPs) are highly abundant and are likely to become the marker of choice in future evolutionary, ecological and conservation studies as genomic sequence information accumulates. As a biallelic marker, SNPs are innately less variable than

microsatellites, but SNPs are the most prevalent form of genetic variation and hence there is a substantial increase in the number of loci available (Brumfield *et al.*, 2003). Furthermore, the simpler mutational dynamics of SNPs lends the advantage of a lowered rate of homoplasy, and, importantly, there is a capacity for rapid, large scale and cost-effective genotyping (Syvänen, 2001).

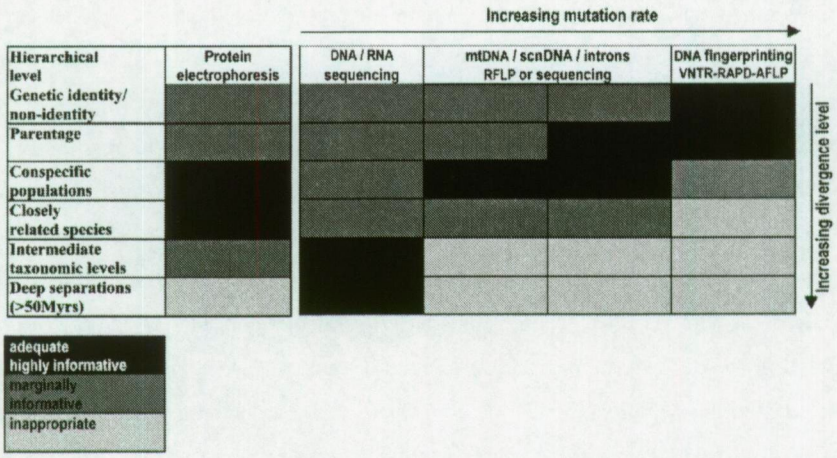


Figure 13 : Choice of molecular markers available to determine genetic relatedness between individuals depending on the level of evolutionary divergence. [AFLP: amplified fragment length polymorphism; mtDNA: mitochondrial DNA; RAPD: random amplified polymorphic DNAs; RFLP: restriction fragment length polymorphism; scnDNA: single copy nuclear DNA; VNTR: variable number of tandem repeats e.g. microsatellite DNA] (Féral, 2002).

Allozyme markers

Protein electrophoresis detects Mendelian variants (allozymes) at identifiable gene loci (Utter *et al.,* 1987). Since electrophoresis is relatively inexpensive, several loci can be examined for genetic variation in large sample sizes. Since the 1960s, allozyme electrophoresis has been widely employed in population genetic studies, reaching its largest applicability during the 1980s (Utter *et al.,* 1987). Enzyme electrophoresis has several advantages placing this technique as a persisting source of genotypic information. The procedure is rather simple and cheap, enabling the screening of many individuals and various independent loci in a short time. There is no need for specific development of markers, as enzymatic reactions are universal among all species. Limitations of this method include the requirement of fresh or appropriately frozen tissue samples. In addition, the sampling of tissues from different organs often requires lethal sampling, although in some cases tissue biopsies or blood may provide

usable data. Lethal sampling may not be desirable in threatened populations with low numbers of individuals. In addition, some changes in DNA sequence are masked at the protein level, reducing the level of detectable variation. Some changes in nucleotide sequence do not change the encoded polypeptide (silent substitutions), and some polypeptide changes do not alter the mobility of the protein in an electrophoretic gel (synonymous substitutions) (Utter *et al.,* 1987). Selective neutrality is not always complete, as several studies showed a possible balancing or directional pattern of allozymatic variation (Pogson *et al.,* 1995; Bossart & Prowell, 1998; Lemaire *et al.,* 2000). In a few cases, correlations exist between selected allozyme markers and performance traits (Hallerman *et al.,* 1986; McGoldrick & Hedgecock, 1997). The detection of selective influences can be of interest when studying species in highly variable environments, but a comparative analysis with a strictly neutral marker (such as microsatellites) ought to be done.

Microsatellite markers

Microsatellites consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs (e.g., ACA or GATA; Tautz, 1989). They vary in the number of tandem repeats, so that they belong to the so called "VNTRs" or Variable Number of Tandem Repeats, and correspond to non-coding regions of the nuclear DNA. Abundant in all species, microsatellites have been estimated to occur as often as once every 10 kb in fishes (O'Connell & Wright, 1997). Microsatellites tend to be evenly distributed in the genome on all chromosomes and all regions of the chromosome. They have been found inside gene coding regions, introns, and in the non-gene sequences (O'Connell & Wright, 1997). Most microsatellite loci are relatively small, ranging from a few to a few hundred repeats, which is important for PCR-facilitated genotyping. Generally speaking, microsatellites containing a larger number of repeats are more polymorphic, although polymorphism has been observed in microsatellites with as few as five repeats (O'Connell & Wright, 1997). Microsatellite polymorphism is based on size differences due to varying numbers of repeat units contained by alleles at a given locus. Microsatellite mutation rates have been reported as high as 10^{-2} per generation (Weber & Wong, 1993), and are believed to be caused by polymerase slippage during DNA replication, resulting in differences in the number of repeat units (Tautz, 1989). Direct studies of human families have shown that new microsatellite mutations usually differed from the parental allele by only one or two repeats (Weber & Wong, 1993), favoring a stepwise mutation model (reviewed in Estoup *et al.*,

2002). However, in a few fish species, alleles with very large differences in repeat numbers have been observed, more in line with an infinite allele model (Balloux & Lugon-Moulin, 2002). Advantages of microsatellites include: a Mendelian fashion of inheritance as codominant markers, high abundance, even genomic distribution, small locus size, and high level of polymorphism. Microsatellites recently have become an extremely popular marker in a wide variety of fields, such as forensics, kinship and parentage analysis, population genetic studies and fisheries research (reviewed in O'Connell & Wright, 1997). This marker class has a stronger discriminative potential for subtle genetic differentiation, considering only drift and mutation as evolutionary forces acting on their evolution (no selection). Moreover, primers developed for one species can usually be used in other related species (Bruford & Wayne, 1993; Roques *et al.*, 2001). One disadvantage of microsatellites is the presence of null alleles that originate through a mutation in the flanking region, and which can be a serious problem in population genetic studies since they induce a strong heterozygote deficit as only one of the two alleles is amplified. Nevertheless, specific tests are available at present in order to correct for such bias.

Classical versus advanced population genetic methods

Population genetics is the field of research looking at the genetic composition of natural populations following Mendel's laws. As opposed to the ideal infinite size population model defined by Wright (1931), natural populations mostly violate many assumptions of classical population genetic models (Hartl & Clark, 1997). The analysis of populations and subpopulations is usually done by partitioning the genetic variability into a within and between population component. The first component can be characterized using estimates such as observed and expected heterozygosity, mean number of alleles and the level of polymorphism. The second component is usually quantified by assessing the variance in genotype or allele frequencies between populations. A basic estimator for such analyses is part of Wright's (1931) *F*-statistics and is defined as F_{ST}. While F_{ST} will continue to be used for comparisons in population genetic studies and as a basic descriptor of population structure (Neigel, 2002), new statistical tools exist now to keep up with the rapidly increasing molecular analysis techniques: it is now possible to infer far more about historical and current demographies of natural populations than can be achieved using traditional approaches (Beerli & Felsenstein, 1999, 2001). In particular, despite being computationally intensive, coalescent-based methods (coalescence theory is the probabilistic description of the

genealogical process for samples of chromosomes in large populations) can estimate several parameters simultaneously, and are not based on summary statistics but instead determine the overall set of parameters that best describe the data. In addition to an estimate of overall population structure comparable to F_{ST}, this parameter set can include estimates of the number of genetically definable subpopulations or clusters in the sample, differentiation estimates for each pair of samples, asymmetrical pairwise gene flow, the relative effects of isolation and migration, current and historical population sizes, and the demographic histories of populations (Wakeley, 1996; Beaumont, 1999; Beerli & Felsenstein, 2001; Nielsen & Wakeley, 2001). Maximum likelihood and Bayesian inference approaches have led to advances in phylogenetic systematics (Lewis, 2001), and these same methods are expanding the analytic power available to population and conservation genetics (Shoemaker *et al.*, 1999).

An increase in genetic data generated by new molecular tools such as microsatellites, sequences, and most recently single nucleotide polymorphisms, has prompted the development of new statistical methods. A central problem for conservation genetics is the identification of evolutionary significant units (ESUs) and management units (MUs). An ESU is a population unit that merits separate management and has a high priority for conservation. ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci. MUs are recognized as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of alleles (Moritz, 1994; Crandall *et al.,* 2000). Traditional estimators rely on the a priori definition of populations, so that their informativeness can be greatly reduced if these pre-defined populations do not accurately describe the biological reality. Several recent methods attempt to circumvent this problem by dividing the total sample into "clusters" of individuals, each of which fits some genetic criterion that defines it as a group (Pritchard *et al.*, 2000; Dawson & Belkhir, 2001). These methods are not coalescent-based, but assign individuals to groups on the basis of their multilocus genotypes, assuming that the markers should roughly be in Hardy-Weinberg and linkage equilibrium within each randomly mating subpopulation. In a sample partitioning method, the goal is not to assign unknown individuals to known populations, but to divide the total sample of genotypes into an unknown number of subpopulations. The most widely used genotypic clustering method thus far is implemented in the program STRUCTURE (Pritchard *et al.*, 2000). This Bayesian clustering method takes a sample of genotypes and uses the assumption of Hardy-Weinberg and linkage equilibrium within subpopulations to find (1) the number of

populations, K, that best fit the data, and, (2) the individual assignments that minimize Hardy-Weinberg and linkage disequilibrium in these populations. Without prior information, STRUCTURE estimates the number of subpopulations, each of which contains a set of individual genotypes in Hardy-Weinberg equilibrium. The data define the populations rather than making best-guess definitions of populations prior to the analysis of genetic structure or using sampling location as a surrogate for the genetic population definition. Furthermore, if all potential source populations have been sampled, the probability output of STRUCTURE can act as a fully Bayesian assignment test for unknown individuals (Manel *et al.* 2002). Individuals of hybrid origin can be assigned proportionally to two or more populations. The identification of hybrid individuals is often a necessary first step in the implementation of management strategies, such as breeding or translocation programs for threatened species (Allendorf *et al.*, 2001) and the standard assignment methods have been applied to detect admixed individuals (Roques *et al.*, 2001; Nielsen *et al.*, 2003; Manel *et al.*, 2005). When parental taxa are not defined or characterized a priori, clustering methods such as the program STRUCTURE (Pritchard *et al.*, 2000) must be used. Anderson and Thompson (2002) developed a method designed specifically to detect hybrids. Unlike methods that estimate the proportion of an individual genome that originated from each taxon (Pritchard *et al.*, 2000), this approach distinguishes various hybrid classes (F1, F2 and various backcrosses). To date, performance of the two types of method has not been compared. Both are appropriate for identifying purebred individuals; however, if distinguishing various hybrid classes is important, the method of Anderson & Thompson (2002) would be more useful.

Assignment methods

When the original population of an individual is unknown, individual assignment tests can help detecting its most likely original population. Assignment tests are based on calculations of the likelihood of multilocus genotypes in populations, based on allele frequencies, and allow the detection of migrants, hybrids and translocated individuals (Hansen *et al.*, 2001). Cornuet *et al.* (1999) developed an assignment test, as well as a modification that statistically excludes potential source populations for a given individual rather than attempting to assign the individual to a source population (as implemented in the software GENECLASS). This can be adequate when not all potential source populations are included in the sampling. Two types of design can be conducted in classical assignment methods: (1) self-assignment, when all individuals from all samples are assigned; thus, an individual may be assigned to the

sample it was derived from or to another sample, and, (2) assignment of unknown individuals, when individuals of unknown origin are assigned to a set of baseline samples (Hansen *et al.*, 2001). Manel *et al.* (2002) compared the performance of partially and full Bayesian methods. The use of STRUCTURE as an assignment test has the advantage that it considers all individuals and populations simultaneously, rather than testing each individual separately for evidence of migrant genetic signal, and such approach improves the power to detect multiple migrants. However, STRUCTURE assumes that all potential source populations have been sampled, and the lack of this requirement in GENECLASS is a significant advantage.

7. Genetic knowledge of the North Atlantic eels *Anguilla anguilla* and *A. rostrata*

Phylogeny of Anguilla *spp.*

Based on a combination of morphological and molecular traits, a total of 15 species have been officially recognized within the genus *Anguilla* (Aoyama *et al.*, 2001, Minegishi *et al.*, 2005). The genus and other Anguillidae families belong together with the Muraenidae to the order Anguilliformes. Recent phylogenetic analyses show that *Anguilla* is a monophyletic group and that the most likely ancestor is *A. mossambica*, which likely originated close to the Indonesian archepelago during the Eocene or earlier (Figure 14). Complex dispersal and subsequent speciation patterns lead to the present worldwide distribution of eel (Aoyama *et al.*, 2001; Lin *et al.*, 2001; Minegishi *et al.*, 2005) (Figure 15).

There are only two species in the North-Atlantic Ocean, the European (*A. anguilla*) and the American eel (*A. rostrata*). Several scenarios have been proposed for their origin, based on fossil records, plate tectonics, paleo-currents and a standard fish molecular clock. A first scenario is the dispersal of ancestral organisms through the Tethys Sea (Figure 16) that separated 70 million years ago Laurasia (North-America and Eurasia) from Gondwana (South America, Australia, Africa and India). Along this sea, dispersal was possible through westerly paleocircumglobal equatorial currents (Aoyama & Tsukamoto, 1997; Aoyama *et al.*, 2001). Aoyama *et al.* (2001) suggested that *Anguilla* speciation started 43.5 Mya and that the North-Atlantic eels speciated some 10 Mya. Although such results were partially confirmed by another study (Bastrop *et al.*, 2000), Lin *et al.* (2001), using a much larger fragment of the mitochondrial genome (cytochrome *b* and 12sRNA), proposed that the genus *Anguilla* speciated much more recently, some 20 Mya.

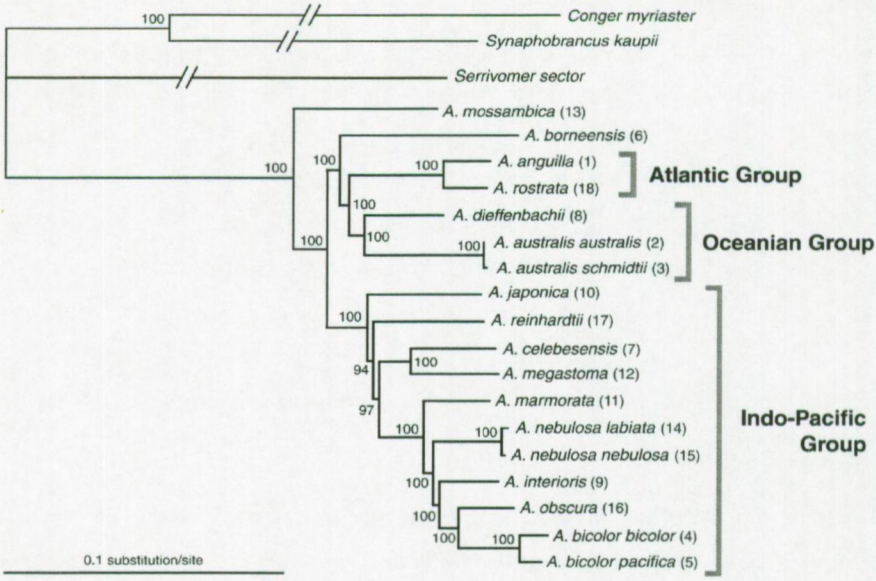


Figure 14 : Current phylogenetic knowledge on the evolution of the genus *Anguilla* based on the complete mitochondrial genome from all 18 species and subspecies (Minegishi *et al.*, 2005).

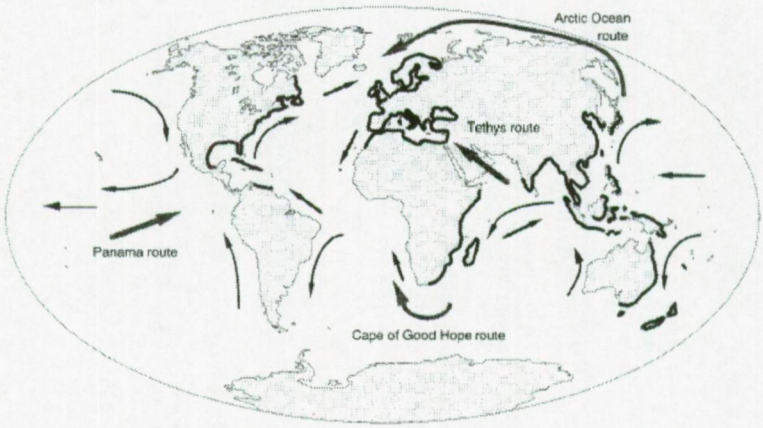


Figure 15 : Present day worldwide distribution of the *Anguilla* spp and the possible dispersal routes (Aoyama *et al.*, 2001).

This study hypothesized that the Atlantic eels colonized the North Atlantic through the Central American Isthmus (Panama) and speciated some 3 Mya (Figure 17). Although these authors used a longer fragment and their speciation estimates are much more congruent with the accepted molecular clock, some incongruence remained. The absence of any eel species on the West coast of North-America or South America and the large phylogenetic distance

with *A. japonica*, who should under this scenario be the ancestor of the North-Atlantic eels, suggest that the radiation events are much more complicated than expected using present day current and tectonic knowledge. A recent study analyzing the complete mitochondrial genome gave additional support for the first hypothesis' dispersal route, but for the second hypothesis' speciation time (Minegishi *et al.,* 2005). Speciation started 20 MyA and formed two main clades, the Atlantic-Oceanian group and the Indo-Pacific group. The present day geographical distribution does not seem to follow phylogenetic relationships anymore in the former, but does so in the latter group (Minegishi *et al.,* 2005). Nuclear data might be the next step to clarify these ambiguities. These results also confirm the instability of morphological characters to discriminate the evolutionary relationships between *Anguilla* species, even after a thorough revision (Ege, 1939; Watanabe *et al.*, 2004).

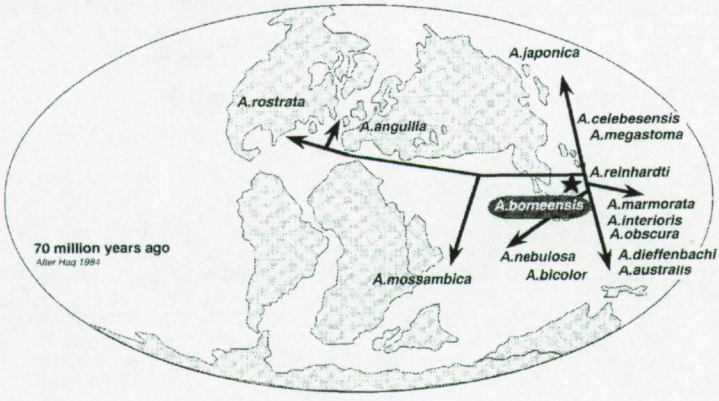


Figure 16: The Thetys Corridor hypothesis speciation scenario of the North-Atlantic *Anguilla* species, formulated in Aoyama *et al.* (2001).

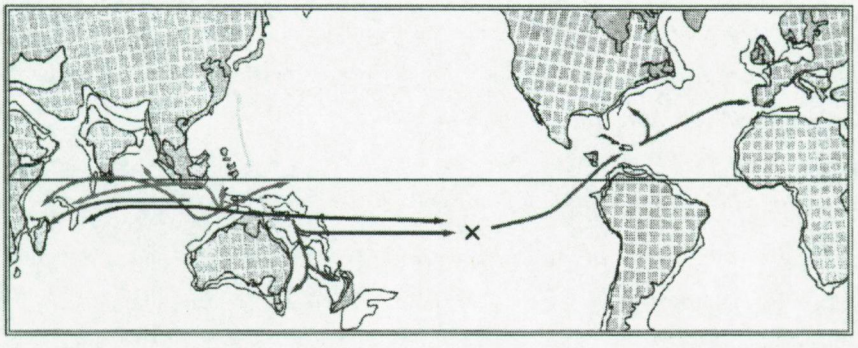


Figure 17 : The Panama hypothesis speciation scenario of the North-Atlantic *Anguilla* species formulated in Lin *et al.* (2001).

The divergence between both North-Atlantic species has been under discussion for decades. Tucker (1959) claimed that differentiating meristic characters (number of vertebrae) were under ecophenotypic selection during the transoceanic migration. The European eel (vertebrae ranging from 110-119, mean 114.7) would be the offspring of the American eel (vertebrae ranging from 103-110, mean 107.1). Tucker (1959) suggested that the European eels do not participate in reproduction, because the distance to the Sargasso Sea was considered too far. Later work, based on variation at hemoglobin, transferrins and allozymes did, however, confirm the two species status (Drilhon *et al.*, 1966, 1967; Fine *et al.*, 1967; de Ligny & Pantelouris, 1973; Comparini & Rodino, 1980; Comparini & Scoth, 1982). The allozyme locus *MDH-2** exhibits a nearly fixed difference between both species, although Williams & Koehn (1984) questioned the taxonomic reliability based on only one enzymatic locus. A mitochondrial DNA RFLP study showed conclusive results, separating both species with high confidence at 11 out of 14 restriction endonucleases, although the two nhg-Atlantic species exhibited the lowest genetic distance reported between *Anguilla* species (Avise *et al.*, 1986; Tagliavini *et al.*, 1995; Aoyama & Tsukamoto, 1997; Ishikawa *et al.*, 2004). Another study assessed the North-Atlantic eel speciation process using jointly distributed parasites (Marcogliese & Cone, 1993). They reviewed the "oceanic" and the "vicariance" hypothesis, suggesting that the two species diverged either in sympatry through differential currents or through the influence of the ice sheets during the Pleistocene, respectively. In the first hypothesis, eels were supposed to live along a single coast (American or European) and disperse through changing currents to the opposite side of the Atlantic, with subsequent assortative mating. The second hypothesis states that the ancestor species had a broad continuous distribution, but split into two groups distributed at each side of the Atlantic under the influence of southward Pleistocene glaciations. The vicariance hypothesis seems to be the most likely to explain the present disjunct transcontinental distribution of the parasites in the study, which can only be transmitted horizontally by continental resident individuals living in freshwater (Marcogliese & Cone, 1993).

Hybridisation between the North-Atlantic eels

Reproductive isolation of sympatric species requires very strong pre- and postzygotic barriers under endo- and exogenous selection (Arnold, 2004). The North-Atlantic eels have been found to be almost completely reproductively isolated, with a small fraction of genetic exchange. Iceland is mainly colonized by European eels, although a small proportion of eels exhibit a vertebrae number smaller than 110 (Avise *et al.*, 1990) (Figure 18).

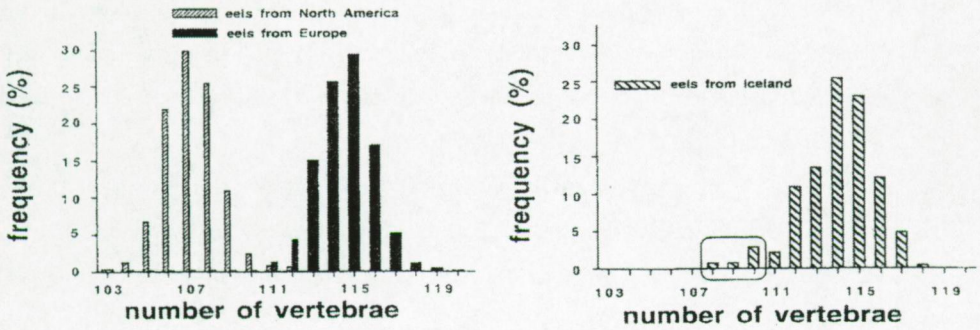


Figure 18: Distribution of the number of vertebrae in the North-Atlantic eels (left). Within the Icelandic "populations", there is a small fraction of eels exhibiting a number of vertebrae characteristic for American eels (from Avise, 2003).

Even though reproductive isolation is strong, indications for hybrids between European and American eel were detected in two studies. Williams & Koehn (1984) compared the *MDH-2** genotypes with the number of vertebrae and concluded that there must be a significant amount of gene flow between both species. Avise *et al.* (1990) evaluated mitochondrial DNA in addition to nuclear and meristic markers in Icelandic individuals. The data reflected cytonuclear disequilibria, most likely due to ongoing gene flow between both species. The study allowed the detection of pure individuals of both species besides hybrids and a quantification of the American eel material in Iceland (2-4%). Recently, Mank & Avise (2003) reassessed these conclusions with highly polymorphic microsatellites markers. Despite the high resolution and power expected from microsatellite markers (Anderson & Thompson, 2002; Manel *et al.*, 2002;), surprisingly no indications for hybridisation were detected (Mank & Avise, 2003). Low sample sizes, the use of basic summary statistics and most likely homoplasy may be the main reasons for the lack of discriminative power between both eel species. This result prompts for further investigations on the paradigm of complete isolation of European and American eels and reopens the debate of the existence and maintenance of a hybrid zone at more than 6,000 km from the spawning site.

Phylogeography and population genetics of the European eel

The phylogeography of the European eel has not been studied that thoroughly (Avise *et al.*, 1986; Lintas *et al.*, 1998; Daemen *et al.*, 2001). Mitochondrial DNA provided only limited insights into the geographical partitioning of genetic variability in European eel, mainly

because of the very high number of haplotypes per sample in the D-loop marker (Lintas *et al.*, 1998). New indications of the non-random distribution of haplotypes were reported using the less variable cytochrome *b* mtDNA marker (Daemen *et al.*, 2001). European eel populations exhibited much lower haplotype diversity at the cytochrome *b* locus compared to the 3' end of the D-loop (Lintas *et al.*, *1998*). The genetic variation observed at the cyt *b* locus was nevertheless high (17 haplotypes in 107 eels), with two central haplotypes in the haplotype network and a significant latitudinal clinal pattern of cyt *b* haplotypes fitting an isolation-by-distance model (Figure 19). There was no indications that the populations of European eel expanded recently.

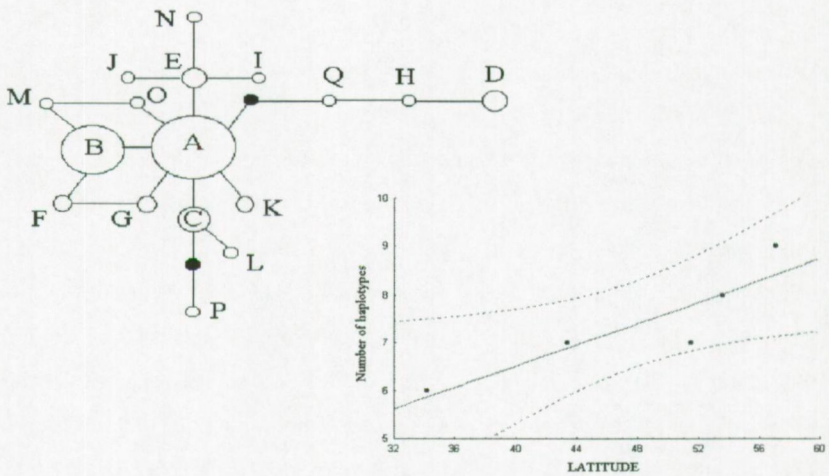


Figure 19: Median Network of all cyt *b* mitochondrial haplotypes detected in European eel and N-S clinal variation in haplotype diversity (Daemen *et al.*, 2001).

For a long time it was assumed that European eel behaved as a panmictic population, i.e. a homogeneous population spawning randomly in the Sargasso Sea. Although, early studies based on allozymes, suggested that European eel populations differed between several continental European locations (Drilhon *et al.*, 1967; Pantelouris *et al.*, 1970), this conclusion was rejected on methodological grounds (Koehn, 1972). Later, allozymatic and mitochondrial DNA studies failed to detect any spatial genetic differentiation (de Ligny & Pantelouris, 1973; Comparini *et al.*, 1977; Comparini & Rodinò, 1980; Yahyaoui *et al.*, 1983; Lintas *et al.*, 1998; Daemen *et al.*, 2001). Similar results were obtained for the American eel (*A. rostrata*) (Avise

et al, 1986) and the Japanese eel (*A. japonica*) (Sang *et al,* 1994), with the exception of clinal allozyme variation putatively imposed by selection in *A. rostrata* (Williams *et al.*, 1973; Koehn & Williams, 1978) and *A. japonica* (Chan *et al.*, 1997). Therefore, panmixia in European eel became widely accepted until two independent recent genetic studies reported evidence for a weak but significant population structure (Daemen *et al.*, 2001; Wirth & Bernatchez, 2001), with the latter study reporting evidence for isolation-by-distance (IBD) (Figure 20). A spatio-temporal differentiation of spawning groups was proposed, to explain such pattern still visible at 6000 km from the spawning site.

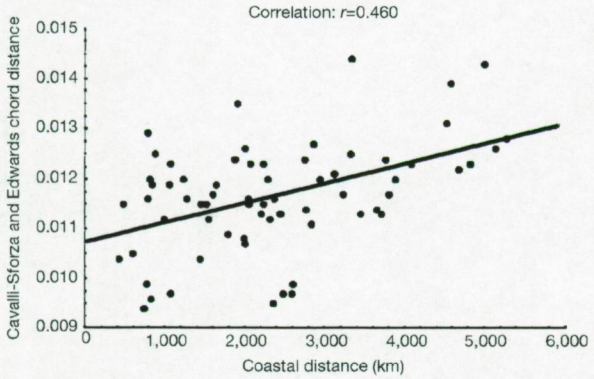


Figure 20: Correlation between genetic distance (D_{CE}) and coastal distance in the European eel as detected in Wirth & Bernatchez (2001).

Genetic knowledge on other Anguilliformes

All *Anguilla* species have a similar life history adapted to oceanic currents and gyral systems (Tsukamoto *et al.*, 2002). Their population genetic structure resembles the one detected in the North Atlantic eels, namely very subtle genetic differentiation over a large area. Studies based on allozymes showed a signal of differentiation between recruiting and resident populations in *A. australis* and *A. dieffenbachii* (Smith *et al.*, 2001). The Japanese eel did not show any evidence of genetic structure over large geographic area in a study on mitochondrial DNA (Sang *et al.*, 1994; Ishikawa *et al.*, 2001). However, in the giant mottled eel (*A. marmorata*) several genetically isolated populations could be detected using mtDNA (Figure 21a) (Ishikawa *et al.*, 2004). Intra-specific divergence was of the same level as the lowest inter-specific divergence in the genus *Anguilla* between the North-Atlantic eels or between the sub-species of *A. bicolor* (Figure 21b). The distribution pattern of five populations was closely associated with the water-mass structure of oceans and major current systems.

a) b)

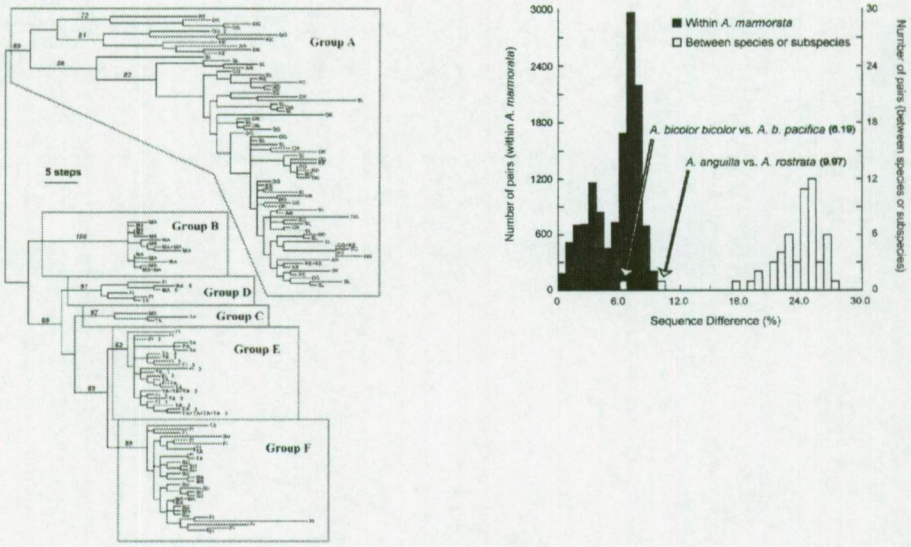


Figure 21 : a) Maximum parsimony trees derived from mtDNA control region sequences from 162 individuals of *Anguilla marmorata*. Numbers above branches show bootstrap values. Upper case letters at the tips of the branches denote collection localities. b) Distribution of pairwise sequence differences in the control region of the mtDNA between individuals of *A. marmorata* and also between ten other species or subspecies of *Anguilla* (Ishikawa *et al.*, 2004).

This observation suggests that present population differentiation in *A. marmorata* might have resulted from the establishment of new population specific spawning sites in different oceanic current systems as the species colonized new areas (Tsukamoto *et al.*, 2002; Ishikawa *et al.*, 2004) (Figure 22). Based on characteristics of the known life history of anguillid eels (e.g., long larval duration and ability to migrate to specific spawning areas) and the general concept of migration loops (Tsukamoto *et al.*, 2002), two basic models can be postulated for the process of anguillid speciation (Figure 22). Model I assumes that the expansion of freshwater growth habitats of anguillid eels takes place first and then reproductive isolation follows when their spawning areas become separated spatially or temporally. This model can explain speciation within an ocean current system and may be exemplified by the Atlantic eels.

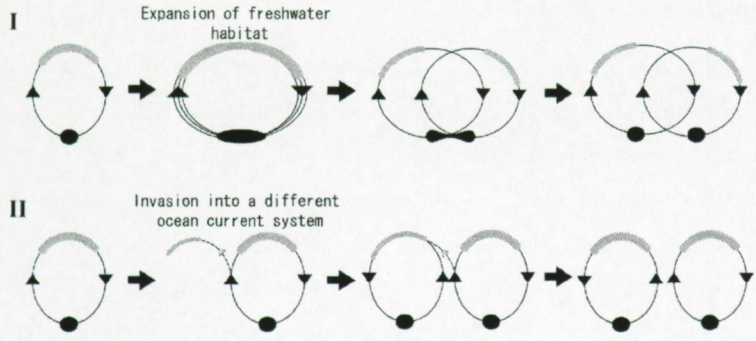


Figure 22 : Two alternative models of the speciation process in anguillid eels. Model I shows speciation within an ocean current system; model II shows speciation beyond the margin of an ocean current system. Circles represent migration loops (Tsukamoto *et al.*, 2002); small solid circles, spawning grounds; shaded arcs, freshwater habitats; lines connecting these with upward arrowheads, transportation of eggs and larvae to the freshwater habitats by an ocean current; lines with downward arrowheads, spawning migrations (Ishikawa *et al.*, 2004).

Alternatively, speciation beyond margins of an ocean current system can be proposed as in model II, assuming that after the expansion of growth habitats, a new spawning site is established first and then (or simultaneously) a separation of growth habitats occurs. The present findings about *A. marmorata* imply that the speciation mode as expressed by model II is plausible in tropical anguillid eels inhabiting adjacent areas.

8. Aims and outline of the thesis

During the completion of its peculiar life-cycle, the European eel is exposed to many endogenous and exogenous selective pressures, such as larval survival, oceanic and climatical forces, migration cues, metamorphoses, freshwater acclimation, intra-specific competition, habitat degradation and pollution, parasite infection, strong fisheries pressure, variance in reproductive success and the need to maintain its species integrity in the sympatric spawning zone with the American eel. In the present thesis, I concentrate my attention on the genetic consequences of the catadromous life-strategy of the European eel. Figure 23 shows its life cycle and at which points in the migration loop genetic alterations can occur. The aims of the present thesis were threefold. (**Part A**) I first assessed the interspecific conservation and species discrimination power of a set of microsatellite markers in several *Anguilla* species, to consecutively focus on the North Atlantic eels, where hybridisation was documented in earlier studies (**Chapter 1 and 2**). (**Part B**) I then tested the spatio-temporal genetic structure of the European eel, combining two sets of molecular markers at a purely spatial scale in adults, a spatio-temporal scale in both juveniles and adults, and a more standardized spatio-temporal scale in recruiting juveniles (**Chapter 3, 4 and 5**). (**Part C**) In the third and last part, I assess the influence of anthropogenic and exogenous stress factors, such as heavy metal pollution and food/space competition in aquaculture, on genetic variability of (sub)adult eels. By analysing the relation between individual heterozygosity and fitness, one may assess the impact of the current population decline on genetic variability and fitness of the European eel (**Chapter 6 and 7**).

In **Chapter 1,** I first evaluate the conservation of the flanking region in microsatellites developed in the two North-Atlantic eel species (*Anguilla anguilla* and *A. rostrata*) in other species, by screening distant Indo-Pacific species for consistency in genetic polymorphism. I then assess the discrimination power of the markers using a Bayesian individual assignment analysis in comparison, to more classical techniques, to provide a tool to detect natural hybridisation and anthropogenic species translocations. In **Chapter 2**, I apply this knowledge to survey several American, European and Icelandic populations for the presence of interspecific hybrids between *A. rostrata* and *A. anguilla*. Using classical and Bayesian assignment analysis tools, I discriminate between several hybrid classes, if present, and test for a link with meristic characters.

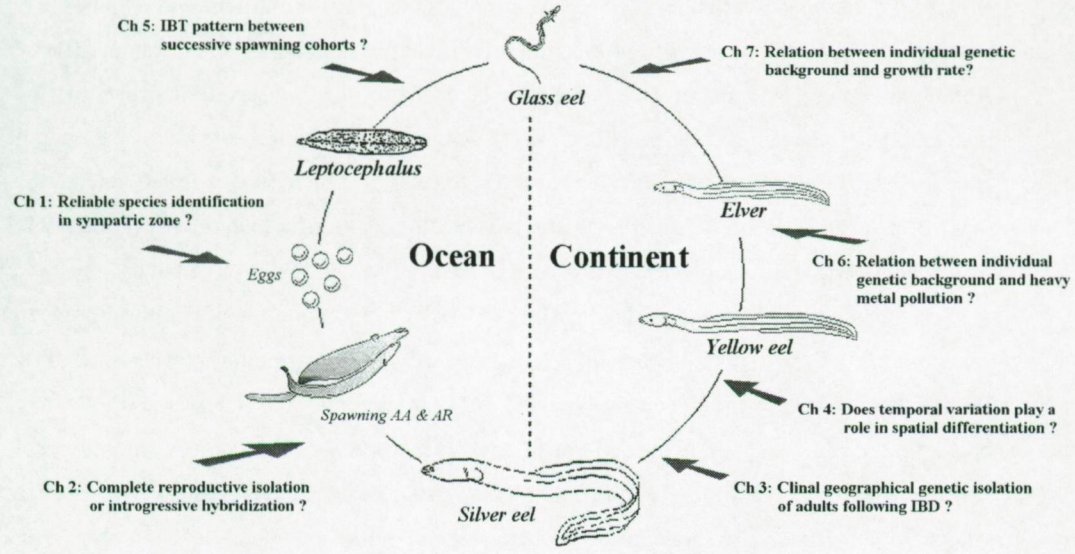


Figure 23 : Life cycle of the European eel and outline of possible genetic consequences of a catadromous migration-loop grouped by chapter.

In **Chapter 3,** I assess the geographical and latitudinal differentiation in the European eel, by sampling (sub)adult eel at several distant localities and by using a higher number of allozymatic markers than in previous studies. I test the long existing paradigm of panmixia within the European eel, by an in depth analysis of the geographical component. To control for temporal variation, I then focus on the spatio-temporal genetic structure over a vast area, by sampling various life stages over several years. In this analysis, I aimed at partitioning the total genetic variation further into a geographical and temporal component, using highly variable microsatellite loci and by sampling extensively within the Mediterranean and the Atlantic Ocean (**Chapter 4**). I then wanted to analyse the temporal variation in greater detail, to reduce the possible genetic noise introduced by the analysis of mixed adult cohorts and the differences in sampling timing at different geographical locations. I therefore sampled "pure" recruiting glass eel cohorts at six well-defined locations during a period of three years. I tested whether temporal variation can be assigned to a stable and continuous isolation in time of genetically distinct cohorts or to an unpatterned variance in reproductive success among

spawning cohorts (genetic patchiness) (**Chapter 5**). By continuously increasing the spatio-temporal sampling scheme in the former three chapters, the insights of the population structure of European eel shifted from a geographical separation following an Isolation-by-Distance (IBD) pattern to a temporal separation following an Isolation-by-Time pattern (IBT). Although various patterns of Isolation-by-Distance have been observed between marine populations, if spawning time is a function of geographical location, temporal and spatial isolation can easily be confounded or misinterpreted if not specifically tested for. If the overlap in spawning time between populations is small, a pattern of Isolation-by-Time (IBT) may be generated between consecutive spawning cohorts, which was observed in chapter 5.

The genetic consequences of the catastrophic decline in population size in the European eel are not known. Population reductions may induce severe population crashes in marine fish, as they suffer from high genetic load and are very susceptible to a decrease in effective population size. In chapters 6 and 7, I verified whether there is a relation between individual genetic variability and fitness in European eel. First, I screened individuals, which were known to be exposed to high doses of heavy metal pollutants, to assess whether river pollutants can hamper survival and reduce genetic variability in natural populations (**Chapter 6**). Using individuals grown in aquaculture, I then tested whether more heterozygous individuals have a selective advantage or higher fitness than more homozygous individuals, by looking at the relationship between individual heterozygosity and length and weight increase (**Chapter 7**).

At the end of this thesis, I combine the obtained results into an evolutionary path shaping the life history of North-Atlantic eels, by critically reviewing the consequences of a catadromous life-strategy on the genetic structure of this "mysterious" fish.

PART A:

Species identification and hybridisation in the genus *Anguilla*

CHAPTER I

Microsatellite conservation and Bayesian individual assignment in four *Anguilla* species

Maes G.E., Pujolar J.M., Raeymaekers J.A.M., Dannewitz J. and Volckaert F.A.M.

SUMMARY

Microsatellite flanking regions are thought to be highly conserved in fish taxa, enabling their application in other species within or outside the source family. However, microsatellite based phylogenetic reconstructions remain doubtful due to allele size homoplasy. Species identification using multi-locus genotypes may suffer less from this phenomenon, when using moderately variable markers. We evaluated the degree of conservation of microsatellite flanking regions and level of polymorphism in relation to phylogenetic distance in four eel species (*Anguilla anguilla, A. rostrata, A. japonica* and *A. marmorata*). Using multiplex PCR reactions developed for the first two taxa, we assessed the discrimination power of an individual based assignment method to differentiate all four species without prior information. Detection and classification of each species was performed with high confidence (> 90 %), as well as assignment of randomly sampled individuals to pre-defined species (> 95%). Our results demonstrate the highly conserved nature of microsatellites and their level of polymorphism in *Anguilla* species, and the power to discriminate between locus-specific and population dynamic effects on genetic variability. Although an inverse relationship was found between genetic diversity and differentiation estimates due to homoplasy, assignment proved to be superior to multivariate and distance based approaches. The method enables the rapid screening of the species status of morphologically similar juveniles and adults using only four loci and the detection of natural hybridization or anthropogenic mixing between internationally traded species.

Keywords: admixture, anguillids, flanking-region, genetic variability, homoplasy, multiplex-PCR, species identification

This chapter is presented as an unpublished manuscript.

INTRODUCTION

Species identification may be based on several methods, ranging from morphology, protein blotting and allozyme scoring to PCR based molecular techniques (Lockley & Bardsley, 2000; Avise, 2004). Morphological analysis, although very useful, lacks power in case of cryptic speciation with homoplasic traits or in species with a large overlap in morphological and meristic traits due to convergent selection (Fisher, 2000; Quinteiro *et al.*, 2001; Jarman *et al.*, 2002). Similarly, protein based techniques, widely used for species identification, lack power to discriminate between related species (Lockley & Bardsley, 2000, Avise, 2004). With the advent of PCR based techniques (such as sequencing, RFLP, AFLP and RAPD), a multitude of taxonomic uncertainties as well as technical problems have been solved. In the first place, molecular analysis at the DNA level has tremendously increased the power to differentiate (cryptic) species, by focusing on the neutral signal of drift and mutation, without appreciable environmental influences (Avise, 2004). By making the distinction between homology (traits which are identical by descent) and analogy (traits which evolved independently multiple times), molecular studies have been able to discriminate between highly related species, such as the species flock of African cichlids (Kocher, 2004). A second advantage is the small size of many PCR-based amplification products, such as PCR-Single Strand Conformational Polymorphisms (SSCP), Single Nucleotide Polymorphisms (SNP) or Simple Sequence Repeats (SSR), enabling the analysis of highly degraded, processed or archival tissue (Buonaccorsi *et al.*, 2001; Jerome *et al.*, 2003; Lucchini *et al.*, 2004).

Mitochondrial DNA (mtDNA) has been the marker of choice for taxonomical questions (Avise, 2004). Nuclear DNA (nDNA) has however several advantages over maternally inherited and haploid mtDNA, such as the capacity to detect biparentally inherited polymorphisms and recent/past species admixture (Ludwig *et al.*, 2003; Lucchini *et al.*, 2004). Microsatellites might be good candidates for identification purposes due to their high variability, codominant diploid inheritance, high intraspecific discrimination power at small and large geographical levels and, most importantly, cross-species amplification (Rico *et al.*, 1996; Roques *et al.*, 2001; Manel *et al.*, 2002). They may be useful to detect jointly cryptic species and intraspecific relationships, but uncertainty remains about their utility for phylogenetic analyses because of homoplasy (Fisher *et al.*, 2000, Estoup *et al.*, 2002). Due to constraints in size, microsatellite alleles will back-mutate to a former state, resulting in alleles identical in state but not by descent. This limits the genetic distance that can accrue between genetically isolated taxa and may lead to spurious patterns (Garza *et al.*, 1995; Nauta &

Weissing, 1996; Paetkau *et al.*, 1997; Fisher, *et al.*, 2000; Estoup *et al.*, 2002). Homoplasy is expected to be high at marine fish microsatellite loci, due to the high population sizes (low effect of drift) in marine organisms and the high mutation rates of microsatellites. By using moderately variable loci, one may reduce the risk that homoplasy interferes with the signal of speciation (Estoup *et al.*, 2002).

Cross-species amplification between more or less distant species is a consequence of highly conserved microsatellite flanking-regions (Rico *et al.*, 1996; O'Connell & Wright, 1997; Neff *et al.*, 1999; Scribner *et al.*, 2000). Amplification success and single locus polymorphism between species is believed to be inversely correlated with phylogenetic distance (Estoup *et al.*, 1995; FitzSimmons *et al.*, 1995). In fish species, cross-species amplification success is in general only slightly influenced by interspecific genetic distance (Rico *et al.*, 1996; but see Guillemaud *et al.*, 2000; Yue *et al.*, 2003, Holmen *et al.* (2005). Studies comparing genetic variability of cross-amplified loci in fish are few and all use a small sample set to check for polymorphism (Schrey & Heist, 2002; Williamson *et al.*, 2002). It is important to discriminate between locus-specific and population dynamic processes when comparing species genetic variability.

Given a baseline population of each species, individual based assignment techniques using various microsatellite markers can differentiate between related species with a high level of confidence (Paetkau *et al.*, 1997; Hansen *et al.*, 2001; Manel *et al.*, 2002, 2005). Individual assignment to a population can be conducted with or without prior information of the number of existing populations (Hansen *et al.*, 2001). In the case that the number of populations is not known, Bayesian clustering and assignment methods may search for the most likely number of populations and subsequently assign each individual with high confidence (Pritchard *et al.*, 2000; Manel *et al.*, 2005). This approach has been shown to enable a >95% confidence of correct identification (Paetkau *et al.*, 1997; Hansen *et al.*, 2001; Manel *et al.*, 2005).

All freshwater eels belong to the genus *Anguilla* and are characterized by a catadromous life history, composed of an oceanic and continental phase. They spawn only once in their lifetime (semelparity), and exhibit a long trans-oceanic migration as leptocephalus larvae, by drifting with major currents towards the continent, where they feed for several years. At partial maturity, they continue their "migration loop" as adult silver eels towards the spawning region, located thousands of kilometers from the feeding habitat (Tsukamoto *et al.*, 2002; Tesch, 2003). A total of fifteen *Anguilla* species are officially recognized, although the morphological and meristic characteristics are highly unstable even between phylogenetically

distant species and remain difficult to use for species determination. A recent reassessment of eel morphology resulted in the detection of only four unambiguous groups and the detection of much overlap in formerly accepted morphological characters (Watanabe *et al.*, 2004). Species recognition is even more problematic at the larval stage, where essential traits such as coloring and dentition characters are lacking (Tesch, 2003; Watanabe *et al.*, 2004). Freshwater eels support important fisheries worldwide, and juveniles (glass eels) as well as adults (yellow and silver eels) are harvested (Dekker, 2003; Tesch, 2003). Recent fisheries data indicate a dramatic decline in the recruitment abundance in three important eel species *A. anguilla*, *A. rostrata* and *A. japonica* since the 1980's (Haro *et al.*, 2000; Dekker *et al.*, 2003;Tseng *et al.*, 2003). In 2004, European glass eel recruitment averaged 1 % of its 1960 level and the entire species is believed to be outside safe biological limits. Although global demand of adult eels are largely met by extensive aquaculture, glass eels for consumption and restocking are still entirely dependent on natural recruitment, as profitable artificial reproduction is still lacking (Dekker, 2003). An efficient and reliable identification of species is critical for eel conservation and aquaculture management. Due to far-reaching translocations of non-native species for aquaculture purposes, the natural distribution of species has become disrupted. The most striking example is the observation of European eels which escaped from culture ponds and migrated together with Japanese eel during the spawning migration in the East China Sea (Zhang *et al.*, 1999; Okamura *et al.*, 2002a).

Genetic distances between freshwater eel species ranges from 9 to 26% based on D-loop sequences (Ishikawa *et al.*, 2004), with most species being very distant, while the distance between the North-Atlantic taxa are in the same order of magnitude as that of the subspecies of *A. bicolor*. Eel taxa may be identified using several molecular techniques (Comparini & Rodino, 1980; Tagliavini *et al.*, 1995; Aoyama *et al.*, 2001; Lin *et al.*, 2001; Rehbein *et al.*, 2002; Hwang *et al.*, 2004), but a rapid and reliable test to identify all *Anguilla* sp. simultaneously in processed, historical or alcohol preserved samples without sequencing is not available (Aoyama *et al.*, 2001; Rehbein *et al.*, 2002; Hwang *et al.*, 2004; Minegishi *et al.*, 2005). Microsatellites have been developed for the three commercially most fished species (*A. anguilla*, *A. rostrata* and *A. japonica*) and several of these markers are known to cross-amplify in related eel species (Wirth & Bernatchez, 2001; Mank & Avise, 2003; Dannewitz *et al.*, 2005). Less related species were never considered, however, and no microsatellite based species identification has yet successfully been tested using such markers.

Here we report on the development and validation of microsatellite DNA multiplex PCRs on four eel taxa, the two North-Atlantic species (*A. anguilla*, *A. rostrata*) and two Indo-

Pacific species (*A. japonica*, *A. marmorata*). Our aims were to (1) evaluate cross-species amplification and the degree of conservation of available microsatellite markers in phylogenetically distant eel species, (2) to assess intra- and interspecific genetic variability and differentiation, while correcting for locus-specific effects, (3) to evaluate the relation between allele frequency (F_{ST}) and allele size (R_{ST}) based differentiation estimators and single locus diversity indices (influence of homoplasy); (4) to screen for the most suitable microsatellite markers for a routine PCR assay and finally (5) to test the discrimination power of a Bayesian individual assignment technique with and without prior species knowledge, in comparison to more classical discrimination techniques.

MATERIAL AND METHODS

Sampling - Three American eel (*Anguilla rostrata,* AR) samples and three European eel (*A. anguilla,* AA,) samples were collected from different geographical locations. One Japanese eel (*A. japonica,* AJ) sample and one giant mottled eel (*A. marmorata,* AM) sample were collected in Taiwan (Table 1). Sample sizes ranged from 30 to 60 individuals. Fin or liver tissue were preserved in 100 % ethanol. Species identification was defined based on geographical sampling location and specific morphological traits following Tesch (2003).

DNA purification and microsatellite amplification – Purification of genomic DNA and amplification of microsatellites loci was performed following methods described in Dannewitz *et al.* (2005). We analysed the following eight nuclear microsatellite loci: AAN 01, AAN 03, AAN 05 (Daemen *et al.*, 2001), ANG 151, ANG 075, ARO 054, ARO 063 and ARO 095 (Wirth & Bernatchez, 2001). Loci named "AAN" or "ANG" have *A. anguilla* as source species, while all "ARO" loci originate from *A. rostrata.* Electrophoresis and size determination of alleles was made on a LICOR 4200 automated sequencer (LI-COR, Inc., Lin, USA) using a 6% acrylamide 7 M urea sequencing gel. A molecular ladder (supplied by the manufacturer) was run along with the PCR products, and allele lengths and genotypes were assessed with the GeneImagIR 4.03 software (Scanalytics Inc, Fairfax, USA).

Genetic data analysis - Genetic diversity was evaluated based on genotype and allele frequencies, using the level of polymorphism (0.95 criterion, where a locus is considered polymorphic when the frequency of the most common allele does not exceed 0.95), observed and expected heterozygosity (H_o and H_e) and allelic richness (R) as criteria. Allelic richness, the observed and expected heterozygosity, and the mean allele size per locus between species

were compared using a regression analysis. A t-test for dependent groups was also performed using STATISTICA 6.1 (Statsoft). Deviations from Hardy-Weinberg equilibrium (HWE) were tested using F_{IS} (Weir & Cockerham, 1984) values calculated in GENETIX version 4.05 (Belkhir *et al.*, 1999). Genetic differentiation was characterized using hierarchical F-statistics, theta (θ, Weir & Cockerham, 1984) and R_{ST}-values as implemented in the FSTAT software package version 2.9.3.2 (Goudet *et al.*, 1995). Significance of F_{IS} and multilocus F_{ST} was assessed with permutation tests (1000 replicates). To test for the influence of homoplasy on inter-species genetic differentiation, a regression analysis between single locus genetic differentiation estimators (R_{ST} and F_{ST}) and expected heterozygosity (H_e) was performed using STATISTICA 6.1 (Statsoft). A factorial correspondence analysis was performed in GENETIX (Belkhir *et al.*, 1999) to plot multilocus genotypes of the four species in three dimensions. To compare the performance of distance based and Bayesian assignment techniques, an allele sharing distance (ASD, Bowcock *et al.*, 1994) matrix was computed from a random subset of 20 individuals per species using the microsatellite toolkit (available at http://acer.gen.tcd.ie/~sdepark/ms-toolkit/) and a Neighbour-joining tree was constructed in PHYLIP version 3.6 (Felsenstein *et al.*, 1993). Genetic variability was partitioned into a within species (F_{SC}) and between species component (F_{CT}) using an Analysis of Molecular Variance (AMOVA) in ARLEQUIN version 2.0 (Schneider *et al.*, 2001). The WHICHLOCI 1.0 software (Banks *et al.*, 2003) was used to target the most discriminating loci for species identification, by simulating 1000 new populations (N = 100) based on allele frequencies data of each species. Through trial assignments with loci one at a time, this software ranks loci in terms of their efficiency for correct population assignment and, conversely, their propensity to cause false assignments. Subsequent trials with increasing numbers of loci determines the minimum number of specific loci needed to attain user defined power for population or species assignment. We used the standard 95 % stringency option for all populations as well as the critical population option, where one specific population has to meet the stringency level (95% correct assignment) instead of the complete dataset. The latter option enables a high assignment success of highly and lowly differentiated samples in one subsequent test. A model based clustering algorithm, as implemented in the software STRUCTURE version 2.1 (Pritchard *et al.*, 2000), was used in order to search for the most likely number of groups (species) in the data. This method assumes no prior knowledge about population structure nor species identity. The software organizes individuals into a predefined number of clusters (K), which may represent putative populations or species, and provides log likelihood values for different Ks. These analyses were performed with $1 < K < 10$ to account for population

structure within species, using the non-admixture model in combination with the non-correlated allele frequency model (high differentiation expected between pure species). To avoid bias, a burn in length of 10^4 iterations followed by 10^5 additional Markov Chain Monte Carlo (MCMC) iterations were performed, as recommended by Pritchard *et al.* (2000). Each assessment of *K* was repeated three times to check the repeatability of the results. The most likely K was then used to assign each individual. Assignments scores (*q*) were plotted and the proportion of correctly assigned individuals ($q > 0.80$) was calculated. Finally, a random set of 100 individuals was drawn from the original dataset; the remaining individuals ($N = 299$) were given a species tag based on morphological critreria and used as a prior group in the STRUCTURE analysis (discarding less than 75 % complete genotypes). Subsequently, the unknown samples were probabilistically assigned to one of the four predefined species clusters. Individuals with an assignment score (*q*) lower than 80 % were considered misclassified.

RESULTS

Cross-species amplification and locus-specific polymorphism of Anguilla sp. – All eight microsatellites developed for the North-Atlantic species, *Anguilla anguilla* (AA) and *A. rostrata*, (AR), amplified successfully in *A. japonica* (AJ) and *A. marmorata* (AM). Only one locus was monomorphic in AJ (AAN 01). There was a significant positive correlation between allelic richness ($r = 0.97$, $p < 0.001$), observed heterozygosity ($r = 0.74$, $p < 0.05$) and mean allele length ($r = 0.99$, $p < 0.001$) at each locus between the source species (AA and AR) (Figure 1). None of the t-tests, testing for pairwise differences in genetic diversity indices, was significant. There was a significant correlation between allelic richness ($r = 0.87$, $p < 0.01$), observed heterozygosity ($r = 0.71$, $p < 0.05$) and mean allele length ($r = 0.78$, $p < 0.05$) at each locus between AJ and the source species. Nevertheless, AJ differed significantly in observed heterozygosity from both source species (t-test, $p < 0.05$). There was a significant correlation between allelic richness ($r = 0.89$, $p < 0.01$), observed heterozygosity ($r = 0.68$, ns) and mean allele length ($r = 0.98$, $p < 0.001$) at each locus between AM and the source species. *A. marmorata* differed in observed heterozygosity (t-test, $p < 0.05$) from both source species. There were no significant differences between all four species in mean allele size and no significant differences between both Indo-Pacific species for any of the three variables.

There was no obvious trend of lower allelic richness with increasing mtDNA distance. The Indo-Pacific species exhibited equal or higher allelic diversity values at 4 out of 8 loci. On the other hand, observed and expected heterozygosity differed between source and the

Indo-Pacific species at several loci, but values were similar at the low variable loci (AAN 01, AAN 03 and ANG 075).

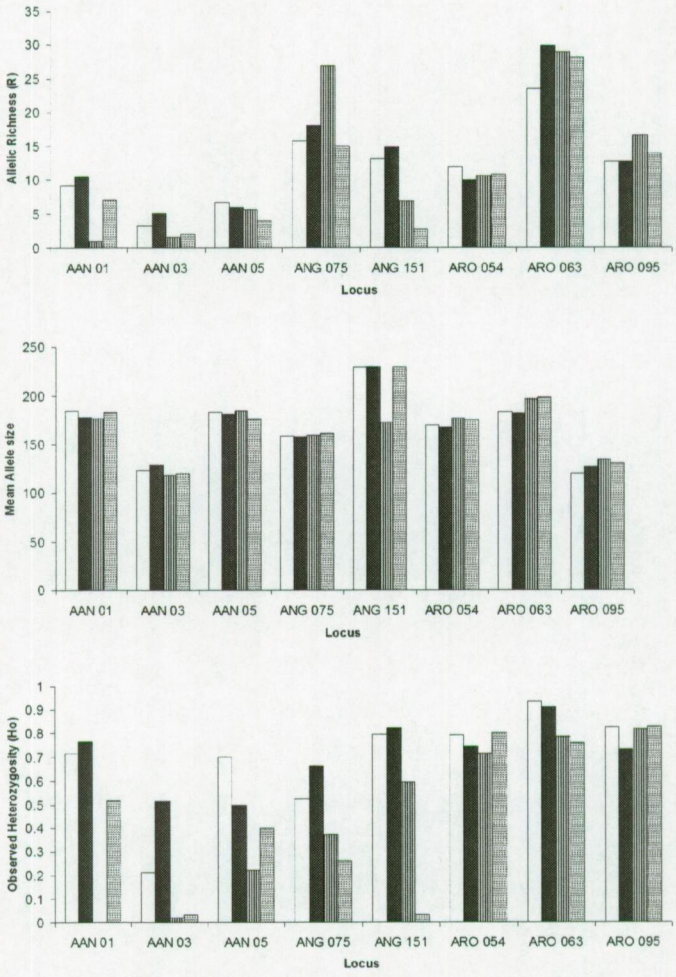


Figure 1 : Comparison among four *Anguilla* species: Allelic Richness per locus (upper panel), mean allele size per locus (middle panel) and observed heterozygosity per locus (lower panel). White bars: *A. anguilla*, black bars: *A. rostrata*, vertical striped bars: *A. japonica*, horizontal striped bars : *A. marmorata*.

Genetic diversity within Anguilla sp. – Genetic variability was high at all 8 loci, ranging from 9 (AAN 03) to 71 alleles (ARO 063). Observed heterozygosity per population and per locus ranged from 2 % (AAN 03 in *A. japonica*) to 93 % (ARO 063 in *A. anguilla*) and H_e per population over all loci ranged from 0.60 to 0.80 (Table 1). Expected and observed

heterozygosity values per locus in a species ranged from 0.22 <H_e< 0.95 and 0.21 <H_o< 0.93 in AA; 0.52 <H_e< 0.96 and 0.50 <H_o< 0.91 in AR, 0 <H_e< 0.95 and 0 <H_o< 0.82 in AJ, 0.03 <H_e< 0.95 and 0.03 <H_o < 0.83 in AM. Allelic richness (R) ranged from 10.42 in AM to 13.42 in AR, with the highest values found in AR and AJ. The difference between observed and expected heterozygosity was mainly due to locus ANG 075, showing strong deviation from HWE and known to exhibit null alleles (Dannewitz *et al.*, 2005). Due to its low influence on genetic differentiation, we decided to keep this locus in the genetic variability and multilocus dataset. On a large scale, the Indo-Pacific taxa (AJ and AM) exhibited a significantly lower genetic variability (allelic richness and heterozygosities) than the North Atlantic taxa (permutation test FSTAT: p = 0.01). Within the North-Atlantic region, no significant differences were observed in genetic variability between species, although AR exhibited higher values for allelic richness and heterozygosities than AA. Within the Indo-Pacific species, AJ exhibited a higher allelic richness, but lower heterozygosities than AM (Table 1).

Table 1 : Sampling details and genetic summary of variability at eight microsatellite loci of four *Anguilla* taxa. Lat = Latitude; Lon = Longitude; Life Stage (LS): G = glass eel; S = silver eel; N = number of samples; H_e: expected heterozygosity; H_o: observed heterozygosity; $P_{(0.95)}$: level of polymorphism at the 95 % level; R: allelic richness.

Species	Sampling site	Lat	Lon	Code	LS	Year	N	H_e	H_o	$P_{(0.95)}$	R
A. anguilla	Burrishoole (Ireland)	53°55′N	09°55′W	AA1	G	2001	60	0.748	0.688	1	11.40
A. anguilla	St Nazaire, Loire (Western-France)	47°12′N	01°44′W	AA2	G	2001	60	0.773	0.725	1	11.86
A. anguilla	Tour-Du-Valat, Rhône (Southern France)	43°33′N	04°38′E	AA3	G	2001	60	0.763	0.648	1	11.86
A. rostrata	St. Johns River Florida (USA)	27°12′N	80°13′W	AR1	S	1999	30	0.779	0.740	1	13.42
A. rostrata	Musquash river New Brunswick	45°11′N	66°19′W	AR2	G	1995	47	0.783	0.690	1	12.22
A. rostrata	West Harbour Pond Maine (USA)	43°59′N	69°50′W	AR3	G	2003	61	0.796	0.702	1	13.27
A. japonica	River Tanshui (Taiwan)	25°10′N	121°25′E	AJ	G	1990	51	0.596	0.441	0.75	12.24
A. marmorata	Shuang-Hsi (Taiwan)	25°01′N	121°51′E	AM	G	1994	30	0.609	0.454	0.87	10.42

Genetic differentiation between Anguilla sp. *and assignment power* – Genetic differentiation was high and significant between all species (overall F_{ST} = 0.19, R_{ST} = 0.26). A locus-by-locus AMOVA analysis confirmed the high interspecific component of genetic variation (18% versus 0.5% between populations within species) (Table 2).

Table 2 : AMOVA: Locus by locus analysis of variance among *Anguilla* species (AS, F_{CT}), within species among populations (WSAP, F_{SC}) and within populations (WP). Global fixation indices per locus are given as F_{ST} and R_{ST}. Most differentiating loci are listed in bold.

** = p-value < 0.01, * = p-value < 0.05

Locus	% AS	% WSAP	% WP	F_{CT}	F_{SC}	F_{ST}	R_{ST}
AAN 01	29.865	0.370	69.764	0.299**	0.0052	0.302**	0.653
AAN 03	62.387	0.183	37.430	0.624**	0.0049	0.626**	0.164
AAN 05	35.105	-0.22	62.118	0.381**	-0.0036	0.379**	0.560
ANG 075	0.975	1.078	97.947	0.009*	0.0109**	0.020**	0.280
ANG 151	15.635	0.069	84.295	0.156**	0.0008	0.157**	0.049
ARO 054	3.711	0.174	96.115	0.037**	0.0018	0.039**	0.005
ARO 063	0.849	1.733	97.418	0.008	0.0175**	0.026**	0.233
ARO 095	1.304	0.606	98.088	0.013*	0.0061**	0.019**	0.062
Total	0.183	0.005	0.812	0.183**	0.006**	0.188**	0.258

The loci exhibiting most differentiation among species were moderately polymorphic, namely AAN 01, AAN 03, AAN 05 and ANG 151 (Table 2). A Factorial Analysis split all genotypes into four distinct clusters based on all eight loci (Figure 2). Both North-Atlantic species are adjacent, while the other two species are much more distinct. The allele sharing distance tree shows four distinct clusters, but individuals from AR are split into two clusters, a separate one and one together with AM (Figure 3). Using only four loci, no clusters are formed (data not shown).

There was a strong negative correlation between F_{ST} and expected heterozygosity ($r = -0.71$ to -0.95, $p < 0.05$), and F_{ST} and Allelic Richness ($r = -0.67$ to -0.82; $p < 0.05$). No significant relationship was found between R_{ST} and any diversity indices. Two loci showed to be the best discriminating between all four species after simulating 1000 new populations using observed allele frequencies. Loci AAN 05 and AAN 01 enabled high assignment success at the 95 % stringency level, with an accuracy of 90.5 % and 87.5 %, respectively. When defining the *A. marmorata* as critical population (stringency of 95% for the least well assigned population instead of over all populations), locus ANG 151 showed to be necessary for correct assignment. The other loci showed a lower discrimination power, locus AAN 03 being highly discriminative between the North-Atlantic species but not in other species (between 46-73%). Further analyses were performed with only the four most discriminating loci (highest F_{CT}) from the AMOVA and WHICHLOCI analysis.

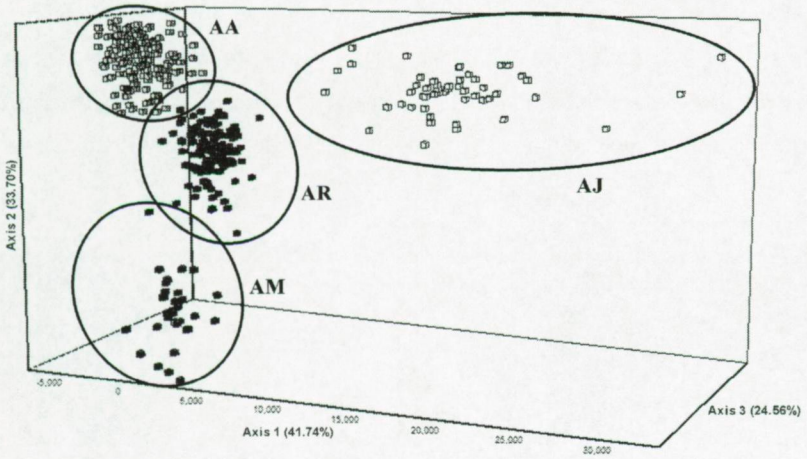


Figure 2 : Factorial Analysis of Correspondence of all *Anguilla* individuals from four species using eight microsatellite loci. Each point represents a multilocus genotype and each color a putative species tag (based on morphology and geographical origin). Light grey = *A. anguilla*; dark grey = *A. rostrata*; white = *A. japonica*; black = *A. marmorata*. Clusters have been circled for clarity.

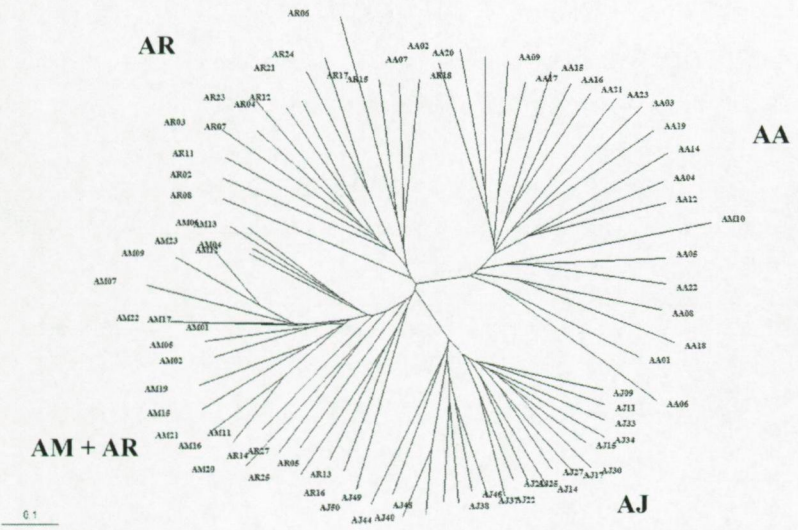


Figure 3 : Allele sharing distance Neighbour-joining tree of a subset of 20 random individuals per species.

Cluster and assignment analysis – The Bayesian MCMC cluster analysis showed a continuous increase in likelihood, but levelled off at K= 4, splitting the dataset into four homogeneous clusters, using four loci. Pritchard *et al* (2000) advise to choose the lowest value when likelihood values are reaching a maximum and then increasing slightly. At K = 5,

the method splits one species (AA) genotypes into two clusters of individuals with equal q-values (0.50), which is highly unlikely. After removal of incomplete genotypes (> 25 % missing), assignment proportions of a putative species to one of the four clusters/species ranged from 0.96 to 1 (Table 3, Figure 4).

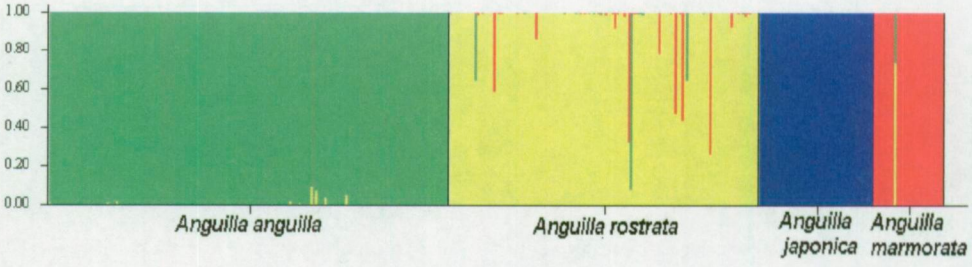


Figure 4 : Results of STRUCTURE clustering and assignment analysis (Pritchard *et al.*, 2000): Bar plot of assignment score for eight populations from four morphologically defined eel species (four colours). Each bar represents one individual. Green = *A. anguilla*; yellow = *A. rostrata*; blue = *A. japonica*; red = *A. marmorata*.

On average 96.5% of all individuals were correctly assigned to their species of origin, except for some AR individuals (9 %). Two individuals were assigned completely or partially to AA, while several individuals showed a signal of admixture with AM (Table 3).

Table 3: Proportion of individuals (morphologically species information known) assigned to each cluster and assignment success a) without prior species knowledge, and b) with randomly drawn eel individuals. An individual is considered correctly assigned if q > 0.80.

	N	Proportion individuals assigned to each cluster				Assignment success (%)
a) Without prior species information		CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 4	
AA	180	1.000	0.000	0.000	0.000	98.25
AR	138	0.012	0.961	0.000	0.026	90.98
AJ	51	0.000	0.000	1.000	0.000	100
AM	30	0.000	0.000	0.000	1.000	96.67
b) Random samples		AA	AR	AJ	AM	
AA-U	36	1.000	0.000	0.000	0.000	100
AR-U	34	0.005	0.973	0.000	0.022	97.10
AJ-U	20	0.000	0.000	1.000	0.000	100
AM-U	10	0.000	0.000	0.000	1.000	100

The accuracy of species identification for a random dataset was tested using as prior baseline data individuals with matching morphological and genetic identity from the first analysis, while assigning unknown individuals to one of the four clusters/species. Most unknown randomly drawn individuals (N = 100) were correctly assigned to their putative species of origin (Table 3). Only AR individuals were not all assigned to their own species (success rate of 97%). One individual showed a *q* score of 0.50 to AR and AM; two other individuals showed a *q* score of 80 %).

DISCUSSION

Conservation of microsatellite flanking region and polymorphism in Anguilla *spp.*

This study shows the highly conserved nature of microsatellite flanking regions of *Anguilla* species, despite their evolutionary distance. All eight microsatellites amplified in the four eel species, with only one locus being monomorphic in Japanese eel. The 100% success in our study is concordant with the observations of Rico *et al.* (1996), who were able to amplify 17 loci (94%) in various fish species based on primers for 18 microsatellite loci originally isolated from whiting (*Merlangius merlangus*), threespine stickleback (*Gasterosteus aculeatus*) and cod (*Gadus morhua*), each representing the three main superclasses and superorders of fish. Levels of polymorphism were generally high in source species or among closely related species. These authors concluded that the conservation of flanking sequences in fish exceeded by far that reported in studies of whales and marine turtles (FitzSimmons *et al.*, 1995). There are several explanations for this high conservation of flanking regions. (1) The rate of base substitution in nuclear and mitochondrial sequences may be lower in fish compared to terrestrial organisms (Rico *et al.*, 1996). (2) The timing of speciation relies on a standard molecular clock and fossil records, and primer site conservation may reflect a more recent origin than expected from mtDNA studies. (3) The supposedly neutral sequences may play an important role in eukaryotic genomes, and may be under strong selective constraints (Rico *et al.*, 1996).

Other studies have reported lower values of cross-species amplification. Holmen *et al.* (2005) tested 120 microsatellite primer combinations developed for *Danio rerio* and *Campostoma anomalum* in seven cyprinids. Amplification products were recorded in only 29-41% of the loci, and 6-23% of the loci exhibited polymorphism. When testing 18 microsatellite loci developed for the catfish *Clarias batrachus* on seven additional catfishes

(Yue *et al.*, 2003), only 50 % cross-amplified in six of the seven species tested. In the latter study the success rate of cross-species amplification varied highly from locus to locus, indicating that cross-species amplification of microsatellites is locus-dependent.

The high-cross species amplification success reported in *Anguilla* is in line with phylogenetic studies, suggesting that the genus has a recent origin. Eel divergence time is still under discussion, ranging from 60 myA to 20 myA considering a standard teleost molecular clock (Aoyama *et al.*, 2001; Lin *et al.*, 2001; Mineguishi *et al.*, 2004). On the other hand, fossil records of the Anguilliformes and the genus *Anguilla* have been found from the Upper Cretaceous (Aptian, about 113–119 Mya) and the Eocene (Ypresian, about 50– 55 Mya), respectively (Mineguishi *et al.*, 2004).

In our study, locus polymorphism was very similar between phylogenetically related or distant species, enabling the direct comparison of variability estimates between loci and species. Genetic variability was high in all species, but there was however a clear difference between the Indo-Pacific and North-Atlantic species. The Indo-Pacific species showed similar diversity values (positive correlation) to the source species, although observed and expected heterozygosity were significantly lower (25 and 20 % differences, respectively). Genetic diversity in our study (mean H_o = 0.45) was lower but of the same order of magnitude than observed for loci developed specifically for AJ (H_o = 0.54-0.89, Tseng *et al.*, 2001; H_o = 0.45-0.86, Ishikawa *et al.*, 2001a). Discrepancies between studies are due to the inclusion in our study of a larger number of moderately polymorphic loci, which are expected to be more adequate for species identification studies. Despite their phylogenetic distance, both AJ and AM exhibit similar and low values, possibly in relation to their demography (smaller *Ne*) or common origin (Mineguishi *et al.*, 2004), rather than to allele length restriction (Garza *et al.*, 1995; Estoup *et al.*, 2002). Population and habitat size, as well as variance in reproductive success of adults are known to influence genetic variability in marine organisms. The stronger effect of genetic drift in small marine populations tends to decrease genetic variability over generations (Hauser *et al.*, 2002; Turner *et al.*, 2002). *A. japonica* is known to have declined dramatically over the last decades (Tseng *et al.*, 2003). Combined with a reduced habitat size (Tesch, 2003), this may have lead to a lower effective population size compared to North-Atlantic eel species. The European eel has indeed the largest "migration" loop among anguillids (Tsukamoto *et al.*, 2002), enabling the maintenance of a larger population size and higher genetic variability than other anguillid species. In the case of the giant mottled eel (*A. marmorata*), its peculiar geographical distribution might play a role, as it is found from East Africa through Indonesia to French Polynesia in the South Pacific Ocean and southern Japan.

Five separate populations have recently been described, suggesting that the species genetic variability might be distributed among various habitats ("migration loops"), with a lower effective population size and thus lower genetic variability per population (Ishikawa *et al.*, 2004).

***The power of individual assignment versus classical methods in* Anguilla spp.**

Our study shows the potential of moderately variable microsatellite DNA markers in discriminating related Anguillid species with a high level of confidence. We provide a powerful tool to identify *Anguilla* species using only four microsatellite loci: AAN 01, AAN 03, AAN 05 and ANG 151. Although the discrimination power for genetic assignment increases with the number of loci used (Hansen *et al.*, 2001), some loci may not provide any discriminatory information due to the lack of polymorphism, significant measurement errors, high correlation with other loci (e.g. linkage disequilibrium) or homoplasy. Loci exhibiting homoplasy will introduce more noise in the dataset than increasing assignment power, as they decrease the overall genetic differentiation by exhibiting alleles identical by state, i.e. without a coalescent origin. Locus set reduction also lowers measurement complexity and related costs (e.g. for genotyping new individuals to be classified in the future). In our study, by removing the four loci showing low divergence values (ARO 095, ARO 054, ARO 063 and ANG 075 with $F_{ST} < 5\%$), a false signal of admixture between distant species can be avoided, while retaining a high assignment power.

The influence of homoplasy on genetic differentiation has been shown in several studies on various organisms (Garza *et al.*, 1995; Estoup *et al.*, 2002), including marine fish (O'Reilly *et al.*, 2004). Estoup *et al.* (2002) showed that homoplasy is expected to be the highest when population sizes and mutation rates are high, two characteristics present in marine species. For instance, O'Reilly *et al.* (2004) demonstrated an inverse relationship between overall F_{ST} and single locus heterozygosity values, a pattern attributed to homoplasy at highly variable loci due to length restriction (Garza *et al.*, 1995). We found a significant negative correlation between F_{ST} and expected heterozygosity and between F_{ST} and allelic richness in all four species, which suggests that moderately variable loci retained better the signal of speciation than highly variable ones. No relationship was found between the allele based differentiation estimator (R_{ST}) and genetic variability, possibly due to the higher variance associated with this estimator (Balloux *et al.*, 2000). Classical multivariate and distance methods performed generally poorer than Bayesian methods. Using a Factorial

Correspondence Analysis (FCA), four clusters corresponding to the four species were clearly discriminated. A graphical view, however, does not provide a usable assignment success proportion to enable power assessment. The allele-sharing-distance tree performed even poorer, and although a rough clustering of the four species was provided, part of the AR individuals clustered into the AM group. It illustrates some shortcomings of these methods, such as the absence of distinctive groups without the information of heir origin and the lack of a statistical framework for individuals misclassified by chance or actively migrating between groups (Pritchard *et al.*, 2000). Another important shortcoming is the impossibility to use prior information gathered on a species/population to help classify unknown individuals into pre-defined clusters. The latter is only feasible using a Bayesian approach (Pritchard *et al.*, 2000).

Multilocus assignment methods versus single locus identification in* Anguilla *spp.

Our approach is as fast and reliable as former eel identification protocols but enables incorporation of additional knowledge, such as sex-biased interbreeding or hybridisation, and includes a power analysis on species discrimination. No sequencing or cloning is needed, and given a baseline population, assignment scores are extremely high (> 97 %). Earlier studies based on reliable PCR-SSCP techniques required a complete diagnostic marker for each species to classify individuals (Rehbein *et al.*, 2002; Hwang *et al.*, 2004).

Assignment tests have proven to be very powerful in individual and/or population discrimination (Hansen *et al.*, 2001; Manel *et al.*, 2005). One crucial requirement for accurate assignment scores to populations or species is a moderate genetic differentiation (F_{ST} > 5%) (Hansen *et al.*, 2001; Manel *et al.*, 2002). In the present study, a mean F_{ST} of 19 % was largely sufficient to discriminate between species. Using either eight or four loci, the four species could be discriminated with high accuracy, suggesting low interspecific information in the remaining four loci with low F_{ST}. The use of microsatellite loci requires some initial knowledge on genetic variability at each locus, to choose moderately mutating loci, less prone to homoplasy (Estoup *et al.*, 2002). For the same reason, it is preferable to use moderately variable loci to study hybridisation between species. No prior knowledge on species identity is required for *de novo* analyses, so cryptic or unknown species as well as hybrid individuals can easily be found (Fisher, 2000; Pritchard *et al.*, 2000; Manel *et al.*, 2002).

Interestingly, *A. rostrata* seemed to show some affinities with *A. anguilla* and *A. marmorata*, as 1.2 % and 2.6 % of the individuals showed a signal of admixture, respectively.

Possible reasons can be either natural hybridisation between adjacently distributed species or the anthropogenic introduction of a foreign species in the range of indigenous eel species. The first reason is very likely between AA and AR, as genetic leakage between both species has been suggested to occur (Avise *et al.*, 1990; Maes *et al.*, 2005, submitted). Our observation thus reflects the power of our method to demonstrate such subtle introgression. The second reason is unlikely for the AR–AM affinity, because of their distant distribution and the unlikely introduction of AM in American waters. An alternative explanation may be the lack of power to discriminate both species without prior knowledge. However, when using prior baseline information and testing random samples, we obtained assignment score above 95 % (see Table 3), confirming the strength of the Bayesian cluster method when using a baseline population.

Phylogenetic analyses should use microsatellites with caution and only after sequencing identical alleles, given the amount of homoplasy detected here. Microsatellite assignment techniques for species identification are very promising, although a case-to-case assessment should be performed to extrapolate the present results to other species. In eel, despite some indications of introgression, our results were in agreement with morphological and geographical prior information. More than 97% of all individuals were correctly identified using assignment techniques. For species showing no external morphological differences at the adult or larval stage, which is typical for many anguillids, the reliable discrimination of such species opens many possibilities to study hybridisation and translocations in a reliable way. Even though there is a lack of strong intraspecific genetic structure in most anguillid species (Ishikawa *et al.*, 2001b; Wirth & Bernatchez, 2003; Dannewitz *et al.*, 2005; but see Ishikawa *et al.*, 2004), assignment would also allow to rapidly detect anthropogenic translocations or natural hybridisation between sympatric eel species. Highly traded species are "accidentally" released in the natural environment. The example of the European eel occurring for 31 % in some Japanese rivers (Zhang *et al.*, 1999; Okamura *et al.*, 2002; Tesch, 2003) highlights the need for identification of possible hybrids between morphologically almost indistinguishable eel species. Both indigenous and translocated species are known to co-migrate (Okamura *et al.*, 2002) and form viable hybrids in aquaculture (Okamura *et al.*, 2004). The method proposed here may achieve this task rapidly and reliably.

ACKNOWLEDGMENTS

This research was financially supported funded by the project EELREP (Q5RS-2001-01836) funded by the European Union. Samples were kindly provided by R. Poole, J. Reynolds, P.

Elie, A. Crivelli, G. Wippelhauser and J. Aoyama. Helpful comments on earlier versions were given by T. Wirth, E. Nielsen, P. Galbusera, L. De Meester and F. Ollevier. G.E. Maes was supported by an IWT (Institute for the Promotion of Innovation by Science and Technology in Flanders) PhD scholarship. J.M. Pujolar received a postdoctoral fellowship from the Ministerio de Educación, Cultura y Deporte (Spain).

CHAPTER II

A tension hybrid zone between the North Atlantic eels of the genus *Anguilla*

Maes G.E., Pujolar J.M., Jonsson B. and Volckaert F.A.M.

SUMMARY

We reassessed the hypothesis of hybridisation between the partly sympatric European eel (*Anguilla anguilla* L.) and American eel (*A. rostrata*) by testing the joint distribution of microsatellite markers and vertebral numbers. We first characterized genetic variability and differentiation between both species in populations throughout Europe and America using 8 microsatellite polymorphic loci. Using classical population genetic techniques, we screened several populations of *Anguilla* on Iceland, where both species have been reported to co-occur, for indications of introgressive hybridisation with the American eel. Icelandic allelic richness, heterozygosity values and allele frequencies were intermediate between both species. Microsatellite loci, selected as moderately variable to avoid homoplasy, yielded a high genetic differentiation between both species (F_{ST} = 0.14, R_{ST} = 0.11; $p < 0.001$), congruent with earlier mitochondrial DNA studies. Subsequent multivariate and individual based assignment tests separated both species with a high level of confidence (> 95% assignment score). Global admixture proportions in Icelandic eel populations attributed 8% of the genomic material to American eel. Classical as well as model-based Bayesian individual assignment tests detected a total admixture within Iceland of 11-15 %, mainly composed of F_1 hybrids (6.3%) and pure *A. rostrata* (3.8%) individuals. The Total Number of Vertebrae was lower in Iceland and co-varied strongly with the admixture coefficient. Our results suggest a narrow tension zone, with asymmetric introgressive hybridisation towards the European eel. The incomplete/imperfect genetic isolation of eels spawning sympatrically affects additional locations in Europe with cryptic American like genomes. Differential oceanic migration of both species is thought to be safeguarded through reinforcement and selection against hybrids.

Keywords: Anguillids; Atlantic Ocean; Bayesian assignment; homoplasy; introgression; marine organisms; population genetics; sympatric speciation

This chapter is presented as an unpublished manuscript.

INTRODUCTION

Hybridization between individuals from genetically distinct populations or species is a central theme in biology, as it interacts with any genetic species concept and species delineation. Regions where genetically distinct individuals interbreed to form genotypes of mixed origin, named hybrid zones, have been studied in numerous ways in view of speciation, adaptation, and co-evolution (Burke & Arnold, 2001; Anderson & Thompson, 2002; Arnold, 2004). Selection acting in hybrid zones may be either endogenous or exogenous (Barton & Hewitt, 1985; Jiggins and Mallet, 2000). The first type is environment-independent and results from the interaction between alleles and genes from distinct taxa. The second type acts postzygotically and is mediated by differential fitness of parental and hybrid genotypes in various environments (Jiggins and Mallet, 2000; Burke & Arnold, 2001; Seehausen, 2004).

Two models have been proposed to explain the presence of hybrid zones (Barton & Hewitt 1985; Burke & Arnold, 2001). Under the "tension zone" model, hybrids exhibit a lower fitness than both parental species (hybrid inferiority) (Barton & Hewitt, 1985). As a consequence, natural selection acts against hybrids directly or through the process of reinforcement, a much-debated pre-zygotic isolating mechanism between species, decrease the chance of hybrid formation (Servedio, 2004). A binomial distribution is then observed consisting of pure individuals of both species plus occasional F_1 and F_2 hybrids (Jiggins & Mallet, 2000). A tension zone is maintained by a selection-dispersal equilibrium in time and space, resulting in gametic disequilibrium in offspring (Barton & Hewitt, 1985). Ecological divergence between most bimodal hybrid zones suggests that ecology contributes more to speciation than genomic incompatibility. Alternatively, hybrids zones may be due to hybrid superiority (Burke & Arnold, 2001). Heterosis and favorable epistatic gene combinations explain the higher fitness of hybrids in comparison with parental species (hybrid vigor). A unimodal genotype distribution is expected, with intermediate genotypes being predominant over parental genotypes (Jiggins & Mallet 2000). Exogenous selection is believed to play a crucial role in the establishment of fit hybrids. Hybrid matings often occur near the edges of a species range or in marginal habitats, where conditions prevail under which new variants are likely to survive and thrive (Burke and Arnold, 2001).

One issue remains unclear for many hybrid zones, namely whether they have a sympatric origin or is due to subsequent secondary contact (natural or anthropogenic). This knowledge is crucial to define conservation issues for endangered species (Allendorf *et al.*, 2001). This can be detected using mtDNA haplotype phylogenies and timing estimates between species

(e.g. molecular clock or mismatch distributions). In a secondary contact zone, the genetic distance between shared and non-shared haplotypes in both species should be much higher than the average distance between non-shared haplotypes within species. Additionally, vicariance enables the calculation of the most likely divergence time between species and the assessment of complete isolation or not.

Marine organisms exhibit a high dispersal potential, a broad distribution, numerous progeny and the absence of reproductive barriers. All these factors favor cross-species fertilization (Palumbi, 1994). Interspecific and intraspecific hybrid zones are more common in the ocean than initially thought (eg gastropods: Rolan-Alvarez, 1997; Cruz *et al.*, 2004; mussels: Bierne *et al.*, 2002; fish: She *et al.*, 1987; Planes & Doherty, 1997, McMillan, 1999; Nielsen *et al.*, 2003). The stability and size of hybrid zones seems not strongly dependent on the dispersal ability (O'Mullan *et al.*, 2001; Roques *et al.*, 2001), but more so on the forces shaping the fitness of hybrids (hybrid superiority vs tension zone) and the evolutionary potential of the hybrids (Moore, 1977; Barton & Hewitt, 1985; Burke & Arnold, 2001).

There are currently 15 *Anguilla* species recognized, all exhibiting a catadromous life history and highly adapted to oceanic currents to complete their life-cycle (Tsukamoto *et al.*, 2002). This study focuses on the two North Atlantic *Anguilla* species, the European eel (*A. anguilla*) and the American eel (*A. rostrata*) (Anguillidae; Teleostei) (Tesch, 2003). Although both taxa spend most of their lifetime in freshwater systems or estuaries, their early and late life-history is entirely comparable to that of marine organisms. They breed in the Sargasso Sea, between 20°N and 35°N, but show some spatio-temporal reproductive segregation (McCleave *et al.*, 1993) and larval migratory segregation (Arai *et al.*, 2000). Glass eels ascend rivers to feed and mature. After several years, in freshwater partially mature silver eels migrate back to their natal spawning grounds. Here they complete maturation, produce up to 2.10^6 eggs per female and die (Tesch, 2003).

The biological and taxonomic integrity of *Anguilla anguilla* and *A. rostrata* have long been under discussion. The only quasi-diagnostic morphometric marker known to distinguish both species is the number of vertebrae. *A anguilla* exhibits 110-119 (mean = 114.7) vertebrae, while *A. rostrata* exhibits 103-110 (mean = 107.1) vertebrae (Tesch, 2003). Tucker (1959) questioned the use of this trait based on possible selective influences on it. The genetic status of Icelandic eels has gained attention after the observation of a small fraction (0.3 % and 5.6 %, respectively) of eels with a reduced number of vertebrae ($\leq$ 110) in Northern Europe and in Iceland. They were proposed to represent either (1) pure American eel expatriates, (2) pure European eel individuals with ontogenic abnormalities or (3) F_1 hybrids.

Avise *et al.* (1990) detected hybrids in Iceland through cytonuclear disequilibria after combining meristic, mitochondrial and allozyme marker. Their data suggested ongoing gene flow between both species, including pure individuals of both species and hybrids incorporating American eel genomic material from 2 to 4%. Mank & Avise (2003) reassessed these conclusions with highly polymorphic microsatellites markers. No indications for hybridisation were detected in this latter study. A potential problem with this study is that highly polymorphic loci can be prone to homoplasy. In a recent paper, Maes *et al.* (2005, submitted) showed that eel species (including *A. anguilla* and *A. rostrata*) can be discriminated with high confidence based on four moderately variable microsatellite loci. The use of four loci is sufficient to detect various hybrid classes (Boecklen & Howard, 1997). We thus set out to use this four-marker set to reassess the hybrid status of the Icelandic eels.

In this study, we sampled putative *A. rostrata* and *A. anguilla* eel together with Icelandic eels. The objective of this study was threefold: (1) We first reassessed the genetic variability and differentiation between the European and American eel using moderately polymorphic microsatellite markers, hence avoiding homoplasy and enabling a highly reliable discrimination of both North Atlantic eel species. (2) We then tested the hypothesis of random hybridisation or unequal introgression between the American and the European eel at several Icelandic sites using meristic (Total Number of Vertebrae) and microsatellite markers. Under a tension zone model, we should detect a bimodal genotypic distribution of pure individuals of both species and occasional F_1 or F_2 hybrids. Under a hybrid superiority model, first generation hybrids should be common and the genotypic distribution more unimodal. The detection of second generation hybrids would imply assortative mating between hybrids, while backcrosses with one of both species are much more likely. (3) Finally, we discuss the possible causes of the presence of hybrids in Iceland and the nature of the hybrid zone in the Sargasso Sea.

MATERIAL AND METHODS

Material and meristic counts - Samples of adult and juvenile eels were collected from four American (N = 210), 9 Icelandic (N = 342) and 10 European (N = 591) locations. Sample sizes ranged from 6 to 60 individuals (see Table 1 & Figure 1 for details). The eels were captured with fyke nets or electrofishing. A piece of muscle or finclip tissue was sampled and stored in 100% ethanol until processing. Only glass eels were digitally X-ray photographed and the number of vertebrae counted by eye.

Table 1: Location of *Anguilla anguilla* and *A. rostrata* samples taken across Europe and North America; LAT : Latitude; LON : Longitude; *CODE*: sampling code; *N* : number of individuals; Life stages: G : glass eel; Y : yellow eel; S : silver eel.

COUNTRY	SITE	LAT	LON	CODE	STAGE	YEAR	N	SPECIES
Iceland	Vogslækur	64°23′N	21°22′W	IC01	G	2001	60	*A.anguilla* ?
	Vogslækur	64°23′N	21°22′W	IC02	G	2003	52	*A.anguilla* ?
	Reykjavik	64°00′N	21°11′W	IC03	Y	2001	11	*A.anguilla* ?
	Floi	63°50′N	20°40′W	IC05	S	1999	6	*A.anguilla* ?
	Reykhólar A	65°26′N	22°13′W	IC06	YS	2001	45	*A.anguilla* ?
	Reykhólar B	65°26′N	22°12′W	IC07	YS	2001	16	*A.anguilla* ?
	Vatnsdalsá	65°35′N	20°20′W	IC08	YS	2000	32	*A.anguilla* ?
	Vífilsstaðavatn	64°07′N	21°52′W	IC09	YS	2002	60	*A.anguilla* ?
Ireland	Burrishoole	53°55′N	09°55′W	AA01	G	2001	60	*A.anguilla*
				AA02	S	2001	60	*A.anguilla*
Netherlands	Den Oever	53°01′N	05°13′E	AA03	G	2001	60	*A.anguilla*
				AA04	S	2001	60	*A.anguilla*
W France	Loire	47°12′N	01°44′W	AA05	G	2001	60	*A.anguilla*
				AA06	S	2001	60	*A.anguilla*
S France	Tour du	43°33′N	04°38′E	AA07	G	2001	60	*A.anguilla*
	Valat			AA08	S	2001	51	*A.anguilla*
Morocco	Sebou	34°16′N	06°34′W	AA09	G	2001	60	*A.anguilla*
				AA10	Y	2001	60	*A.anguilla*
N-America	Florida	27°12′N	80°13′W	AR01	S	1999	30	*A.rostrata*
	Maine	44°02′N	69°58′W	AR02	G	2003	60	*A.rostrata*
		43°59′N	69°50′W	AR03	G	2003	60	*A.rostrata*
		43°51′N	69°37′W	AR04	G	2003	60	*A.rostrata*

DNA purification and microsatellite amplification – Purification of genomic DNA and amplification of microsatellites loci was performed following methods described in Dannewitz *et al.* (2005). We analysed the following eight nuclear microsatellite loci: AAN 01, AAN 03, AAN 05 (Daemen *et al.*, 2001), ANG 151, ANG 075, ARO 054, ARO 063 and ARO 095 (Wirth & Bernatchez, 2001). Electrophoresis and size determination of alleles was made on a LICOR 4200 automated sequencer (Westburg, Leusden, The Netherlands) using a 6% acrylamide 7 M urea sequencing gel. A molecular ladder (supplied by the manufacturer) was run along with the PCR products, and allele lengths and genotypes were assessed with the GeneImagIR 4.03 software (Scanalytics inc, Fairfax, USA).

Data analysis of genotypes - Genetic diversity estimates such as the level of polymorphism, and observed and expected heterozygosity (H_o and H_e) were calculated in GENETIX version 4.05 (Belkhir *et al.*, 1999). Allelic Richness (R) and Gene Diversity (H_S) comparisons between species (*A. anguilla* – AA and *A. rostrata* AR) and Icelandic samples, as well as

departures from Hardy-Weinberg equilibrium (F_{IS}) and R_{ST} values (Rho ST) were calculated using the software FSTAT version 3.9.5 (Goudet, 1996). Genetic differentiation was characterized using hierarchical F-statistics (θ, Weir & Cockerham, 1984) and G_{ST}-values as implemented in the GENETIX 4.05 software package (Belkhir *et al.*, 1999). Significance was assessed with permutation tests (1000 replicates). In all cases, significance levels were corrected for multiple comparisons using a sequential Bonferroni correction (Rice, 1989). Unbiased pairwise genetic distances (Nei, 1978), a Neighbour-joining dendrogram and branch boostrap values (1000 iterations) were calculated using the software package PHYLIP (Felsenstein, 1996). Due to homoplasy or uninformative loci, misassignments can easily occur or the confidence interval may become very broad, especially when analysing admixed individuals. We used the WHICHLOCI software (Banks *et al.*, 2003) to pinpoint the most discriminating loci between species and avoid possible misclassifications due to homoplasy. The software GENECLASS (Piry *et al.*, 2000) was used to assign individuals to their respective species. We first used a classical Bayesian assignment method (Rannala & Mountain, 1997), assuming known baseline populations without admixture analysis. To assess the species integrity within each species and to detect admixture, a fully Bayesian model based individual clustering algorithm was used, as implemented in the software STRUCTURE 2.1 (Pritchard *et al.*, 2000). This method assumes no prior knowledge about population structure. The program organises individuals into a predefined number of clusters (K), which may represent putative populations or species, and returns log likelihood values for different Ks. Initial analyses were performed with K=1 to K=5, to account for possible population structure within species. The non-admixture model was used in first instance (to detect pure non-admixed genotypes within each species) in combination with the correlated allele frequency model (low genetic differentiation at several loci) to discriminate the two species with high confidence. A burn in length of 10,000 iterations followed by 100,000 additional Monte Carlo iterations were performed as recommended by Pritchard *et al.* (2000). Each assessment of K was repeated three times to check the confidence of the results. The admixture model was then applied with prior morphological and genetic species information and the correlated allele frequencies model to detect admixed genotypes (hybrids) within Icelandic and baseline samples. We considered individual with admixture coefficients (q) between 0.80 and 1 to be a pure species and scores between 0.80 and 0.20 to be hybrids or backcrosses. A recently developed specific test to accurately discriminate between hybrid classes, implemented in the software NEWHYBRIDS version 1.1 (Anderson & Thompson, 2002) was subsequently used. This algorithm can assess fine scale hybridisation between two

species, enabling the detection of six different genotype classes, namely pure species, F_1, F_2 hybrids and backcrosses between hybrids and pure species. The program computes by MCMC the Bayesian posterior likelihood that individuals fall into different hybrid categories (based on allele frequency distributions). To test the discriminative power of all loci versus the most discriminating ones detected before, we repeated this last analysis using both datasets (discarding the non informative loci in the second analysis). We then compared classical assignment (GENECLASS), model based Bayesian assignment (STRUCTURE) and NEWHYBRIDS assignment results to increase the confidence of hybrid detection. Finally, we calculated the parental contribution of both species within the Icelandic group of individuals using the ADMIX software (Bertorelle & Excoffier, 1998), yielding two estimators of admixture proportions based on a coalescent approach that explicitly takes into account gene frequencies and molecular information. We used both the gene frequency (m_R) as the coalescent (m_Y) methods, assuming a large genetic differentiation between both species. Standard deviations were calculated by bootstrapping technique for each parental and hybrid population (1000 bootstraps). The power of detection of hybrid individuals through H&W disequilibrium is low in marine species with high expected heterozygosities (H_E) (Nielsen *et al.*, 2003). Tension zones are often characterized by gametic phase disequilibrium due to dispersal of parental gene combinations into the center of the zone (Barton & Hewitt, 1985). Gametic phase disequilibrium, corresponding to the correlations of gene identities across loci within samples, was estimated to detect admixture and infer the nature of a possible hybrid zone (tension vs. hybrid vigor zone) using the program ESTIM 1.1 (Vitalis & Couvet, 2001).

RESULTS

Meristic characteristics – The Total Number of Vertebrae (TNV) within the American samples (N = 3) ranged from 104 to 110, with a mean of 107.2 ($\pm$ 2.08). The number of vertebrae within the European eel samples (N = 5) ranged from 110 to 119, with a mean of 114.9 ($\pm$ 1.25). The meristic counts of Icelandic samples (N = 2) ranged from 106 to 120, with a mean of 113.6 ($\pm$ 2.17). Out of 107 X-ray photographed Icelandic glass eels, 8 exhibited intermediate TNV counts of 110 or less (7.4 %) compared to 2 out of 296 (0.6 %) in European glass eels (Figure 1).

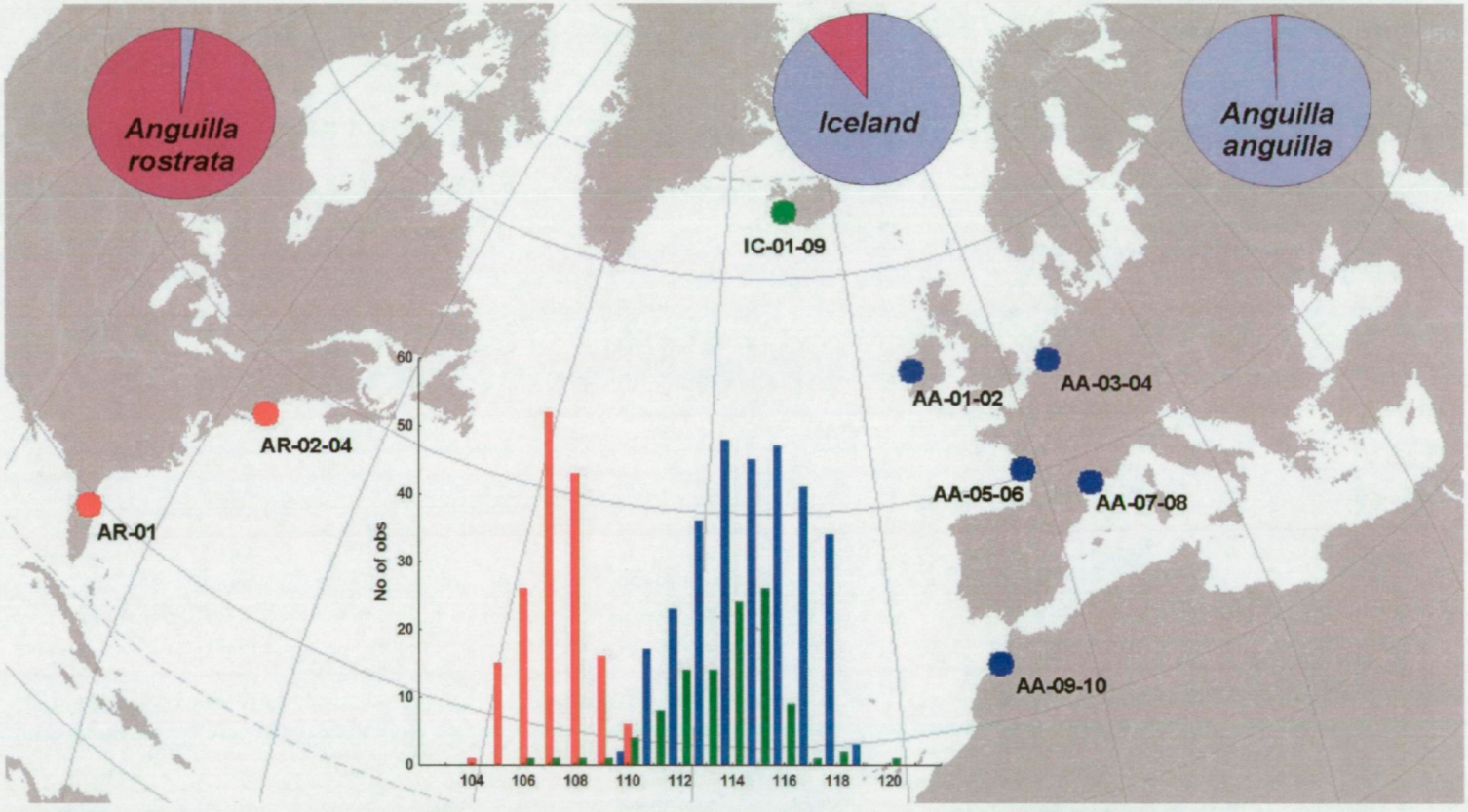


Figure 1: Sampling locations of European eel (*Anguilla anguilla* L., AA-01-10), American eel (*A. rostrata*, AR-01-04) and Icelandic eel (IC-01-09) (for sampling codes, see Table 1). The pie diagram represents the admixture proportion within each species and in Iceland based on combined results of the three assignment methods (Table 3). The histogram represents the Total Number of Vertebrae (TNV) distribution in European eel, American eel and Icelandic individuals.

Genetic variability and departures from Hardy-Weinberg proportions – Genetic variability estimates were similar for European eel (H_E = 0.73-0.77; R = 5.54; H_S = 0.77) and American eel (H_E = 0.78-0.80; R = 5.78; H_S= 0.80). Icelandic samples (IC) mostly exhibited intermediate diversity values: 0.72-0.79 (H_E), 0.79 (H_S) and 5.60 (R) (Table2). All three groups differed significantly in gene diversity and allelic richness amongst each other ($p < 0.05$). Allelic distribution differed highly across loci (Figure 2).

Table 2 : Genetic diversity estimates of *Anguilla anguilla*, *A. rostrata* and Icelandic samples, including expected (H_e) and observed heterozygosity (H_o), Gene diversity (H_s) and allelic richness (R). Standard deviation is showed between brackets.

LOCATION/SPECIES	SAMPLE	$H_{e\cdot}$	$H_{o\cdot}$	H_s	R
Icelandic samples	IC01	0.7685 (0.2118)	0.7297 (0.2016)	0.776	5.574
	IC02	0.7502 (0.2042)	0.6320 (0.2125)	0.760	5.391
	IC03	0.7013 (0.2742)	0.7260 (0.3248)	0.747	5.411
	IC04	0.7474 (0.2369)	0.6657 (0.2292)	0.757	5.390
	IC05	0.7272 (0.1242)	0.6810 (0.1952)	0.811	5.199
	IC06	0.7591 (0.1779)	0.6653 (0.2179)	0.776	5.324
	IC07	0.7703 (0.1235)	0.7946 (0.1759)	0.835	5.778
	IC08	0.7410 (0.2386)	0.6789 (0.2512)	0.756	5.321
	IC09	0.7681 (0.2041)	0.7018 (0.2180)	0.780	5.536
European samples	AA01	0.7241 (0.2813)	0.7139 (0.2645)	0.731	5.190
(Anguilla anguilla)	AA02	0.7411 (0.2509)	0.6268 (0.2110)	0.750	5.351
	AA03	0.7448 (0.2371)	0.6851 (0.2233)	0.752	5.271
	AA04	0.7449 (0.2170)	0.7354 (0.2067)	0.752	5.333
	AA05	0.7562 (0.2224)	0.7267 (0.2032)	0.763	5.423
	AA06	0.7513 (0.2294)	0.7430 (0.2112)	0.759	5.427
	AA07	0.7429 (0.2453)	0.6892 (0.2462)	0.750	5.307
	AA08	0.7192 (0.2819)	0.6746 (0.2629)	0.728	5.216
	AA09	0.7564 (0.1968)	0.7198 (0.1884)	0.764	5.275
	AA10	0.7441 (0.2311)	0.7024 (0.2217)	0.751	5.276
American samples	AR01	0.7580 (0.2077)	0.7405 (0.1738)	0.772	5.519
(Anguilla rostrata)	AR02	0.7796 (0.1744)	0.6713 (0.1794)	0.788	5.530
	AR03	0.7680 (0.2007)	0.6692 (0.1704)	0.776	5.585
	AR04	0.7786 (0.1658)	0.7138 (0.1439)	0.786	5.580

Loci AAN 05, AAN 03, AAN 01, ARO 054 and ARO 063 showed strong differences between species and various species-specific alleles. Locus ANG 075 showed a strong deviation from H&W expectations (F_{IS} > 30%), probably because of the presence of null alleles. As this locus does not provide any discrimination power to separate the two species (see lower), it was removed from the dataset. In the remaining dataset, among 161 tests (23 samples × 7 loci) for HWE, 17 (10 %) showed significant deviations from expected genotype

frequencies after sequential Bonferroni correction (α = 0.05, k = 23). All deviations represented heterozygote deficiencies at the loci ARO 054 and ARO 095. The risk of encountering heterozygote deficiencies as a result of large-allele dropouts has been mentioned to increase when multiplexing primers, especially for highly variable loci (O'Connell & Wright, 1997).

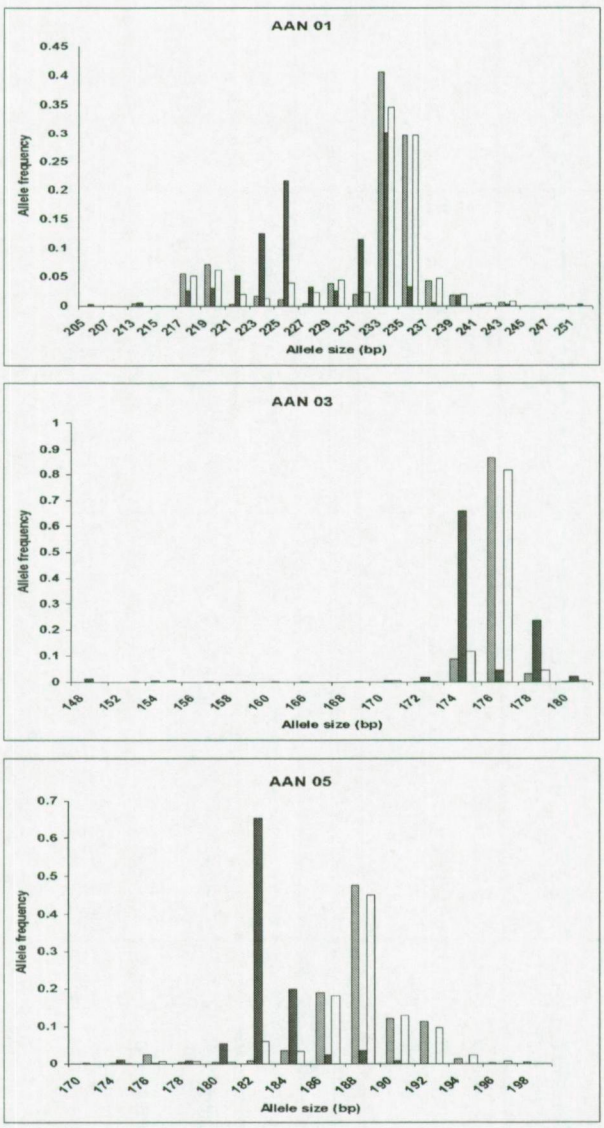


Figure 2: Allelic frequency distribution in *Anguilla anguilla* (light grey bars), *A. rostrata* (dark grey bars) and Icelandic samples (white bars) at the three most discriminative microsatellite loci: AAN 01 (upper panel); AAN 03 (middle panel); AAN 05 (lower panel).

To test for this, we re-amplified the two deviating loci separately in two different samples, but found identical genotypes, ruling out this reason for deviation. We kept to our original dataset because both loci did not influence genetic differentiation values in a previous study on a larger scale (Dannewitz *et al.*, 2005), and because populations from Iceland possibly consisted of admixed individuals. Once corrections for null alleles are applied, genotypic information is lost (Dannewitz *et al.*, 2005). Although not significant, the Icelandic populations exhibited higher F_{IS} values than both species separately. Raw allele frequencies provide further evidence of affinities between both species. Icelandic populations show a decrease of the most common AA allele and an increased frequency of the most common AR allele at loci AAN 01, AAN 03 and AAN 05 (Figure 2). Overall, several Icelandic samples take an intermediate position at the most divergent loci (data not shown).

Multi-locus analysis of genetic differentiation – Overall F_{ST} between all samples was 0.05; global G_{ST} 0.06, while overall R_{ST} amounted to a similar value of 0.04. When considering all samples within species together (removing possibly introgressed Icelandic samples), overall genetic differentiation between species was high (F_{ST} = 0.14, G_{ST} = 0.075 and R_{ST} = 0.11), with a maximum pairwise F_{ST} value of 16.3 % between samples AA1 and AR1. There is no diagnostic difference between species at any single locus, but there are three main differentiating loci: AAN 03 (highest F_{ST}), AAN 05 (highest R_{ST}) and AAN 01. Loci ARO 054, ANG 151 and ARO 063 have the same order of magnitude of differentiation (F_{ST} around 2 %, data not shown). All values were highly significant ($p < 0.001$), except for ANG 075, a locus we discarded in the further individual analyses due to the presence of null alleles and the lack of a differentiation signal. Within species, differentiation was very low ($F_{ST} < 0.01$) and only showed some significant values in the European eel, without any evidence of geographical clustering (data not shown). A phenogram constructed using unbiased Nei (1978) distance, separates *A. rostrata* and *A. anguilla* with high confidence (98%) and places several Icelandic populations at an intermediate position between both species or within the *A. anguilla* cluster (Figure 3).

Power of assignment – In total, 1000 new populations (N = 100) were simulated from each species based on the allele frequencies data to target the most discriminating loci for species identification. One locus (AAN 05) was sufficient to discriminate both species with high accuracy (98.5 %). In order to increase the detection power for F_1 and further hybrid classes, two datasets were constructed. A first dataset included the best classifying loci, namely AAN

05, AAN 03 (94.5 %), AAN 01 (88%) and ARO 063 (76.5 %). A second dataset included all loci (excluding ANG 075). Estimates of correlation of gene identities (eta_S = 0.000832) at pairs of loci (gametic phase disequilibrium) revealed a positive significant outcome (95 % Confidence interval: [0.000281, 0.001920]) in the pooled Icelandic sample, but not in the pure species (eta_S < 0).

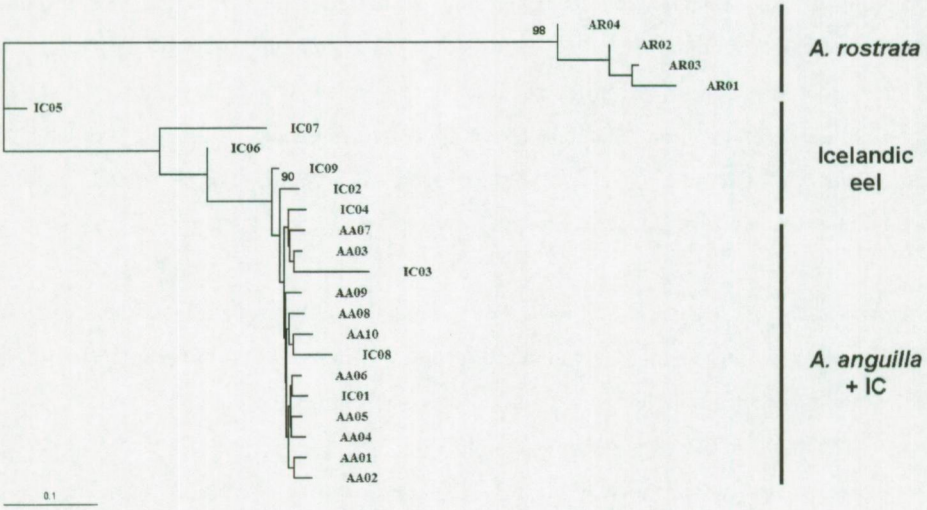
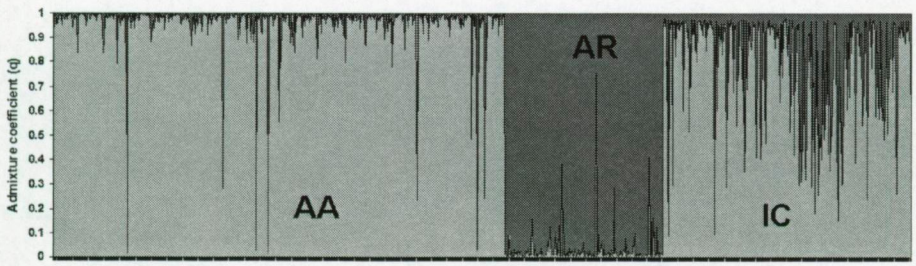


Figure 3: Neighbour-joining tree of all *Anguilla* samples based on pairwise unbiased Nei (1978) distances. Confidence values after 1000 bootstraps.

Detection, extent and level of introgression in Anguilla species - We first used classical assignment to test the discriminative power of the applied method. When both presumably pure species were analysed, more than 99 % of all individuals were correctly assigned to their morphological species class (Table 3). We detected 0.5 % American material (3 pure AR, 3 partially admixed individuals) in the European populations and 1 % European material (2 pure AA, 3 partially admixed individuals) in the American populations. When the Icelandic individuals were treated as an unknown sample to be assigned to one of the two species, 31 individuals (11.9%) with American eel ancestry were detected in the predominantly European Icelandic samples (Table 3). Most (6.9 %) of the AR-like individuals in Iceland showed the pattern of admixture between both species (18 individuals with score < 0.90) and 5 % were considered pure AR (13 individuals with score > 0.90). To differentiate between real hybrids and a lower assignment success of a pure individual, a fully Bayesian approach was implemented in STRUCTURE. Initially, a cluster analysis was performed to test pure

individuals of both species; K = 2 showed the highest posterior probability. Assignment proportions were very high, approximating 99 % in both species (Table 3, Figure 4a). In the European eel, we found four pure AR, two completely admixed individuals and one partially admixed individual. In the American eel, five highly admixed individuals were detected. The Icelandic samples were subsequently analysed as unknown individuals, while both morphological species were given a prior of known species flag. Icelandic samples exhibited a high admixture proportion (85 % AA, 15 % AR) (Figure 4a and 4b). When tentatively defining classes, 78% pure AA ($1.0 < q < 0.80$), 1.1% pure AR ($q < 0.20$) and 21% hybrids ($0.20 < q < 0.80$) were differentiated (Figure 4b). Further geographical analysis of admixture proportions showed that *A. rostrata* material in European populations is not only present in Iceland (11.5 %), but also in Morocco (0.7%) and The Netherlands (0.2%), although only as admixed ancestor (one generation back admixture). There was also a slight difference in admixture proportion between life stage, and among geographical locations within Iceland. Sample IC05 has the highest American eel material, but this sample consisted of already sorted individuals with discordant vertebral count versus mtDNA haplotype (Table 3).

a)



b)

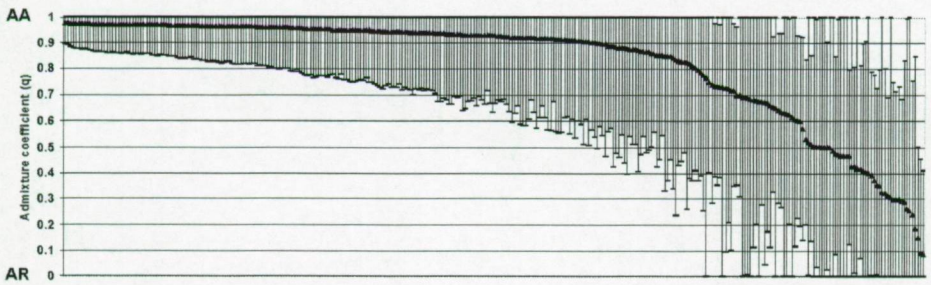


Figure 4: Admixture (STRUCTURE) results of European, Icelandic and American *Anguilla* at K= 2 (2 species). a) Each vertical bar represents the admixture proportion of an individual. Dark grey represents AR, light grey represents AA. Icelandic individuals are represented at the end of the figure. b) Admixture proportion of all Icelandic individuals at K = 2, with confidence interval bars (0.90). $q = 1$ is a pure AA, $q = 0$ is a pure AR.

Table 3: Assignment success (Classical method) and admixture proportion (Model based method) and Hybrid class probability (NewHybrids method) of both pure species and Icelandic eel individuals. AA: *Anguilla anguilla*, AR: *A. rostrata*, IC: Icelandic populations, F_1 : First generation hybrids, F_2 : second generation hybrids, BX AA : Backcrosses between F_1 and AA, BX AR : Backcrosses between F_1 and AR. m_Y and m_R: Estimator of Admixture proportion using gene frequencies (m_R) and coalescent information (m_Y)

	CLASSICAL ASSIGNMENT		MODEL BASED ASSIGNMENT		HYBRID CLASS ASSIGNMENT					
Presumed PURE species	**AA**	**AR**	**AA**	**AR**	**AA**	**AR**	**F_1**	**F_2**	**BX AA**	**BX AR**
AA	0.9950	0.0050	0.9890	0.0110	0.9893	0.0015	0.0079	0.0008	0.0006	0.0001
AR	0.0100	0.9900	0.0090	0.9910	0.0088	0.9649	0.0203	0.0039	0.0002	0.0018
ALL ICELANDIC EELS	0.8810	0.1190	0.8500	0.1500	0.8928	0.0380	0.0626	0.0043	0.0015	0.0008
IC01	0.9091	0.0909	0.8800	0.1197	0.9077	0.0301	0.0578	0.0027	0.0011	0.0005
IC02	0.9375	0.0625	0.8620	0.1381	0.9514	0.0207	0.0233	0.0031	0.0011	0.0004
IC03	0.8889	0.1111	0.8810	0.1188	0.9690	0.0111	0.0128	0.0046	0.0022	0.0003
IC04	0.8913	0.1087	0.8760	0.1239	0.9116	0.0137	0.0702	0.0028	0.0012	0.0005
IC05	0.3333	0.6667	0.5990	0.4013	0.5081	0.2479	0.2193	0.0170	0.0030	0.0047
IC06	0.8077	0.1923	0.7350	0.2649	0.7206	0.0998	0.1638	0.0106	0.0027	0.0024
IC07	0.7143	0.2857	0.6750	0.3250	0.7169	0.2244	0.0486	0.0066	0.0011	0.0024
IC08	0.9259	0.0741	0.8730	0.1271	0.9116	0.0214	0.0606	0.0039	0.0021	0.0004
IC09	0.9429	0.0571	0.8880	0.1116	0.9664	0.0106	0.0182	0.0033	0.0012	0.0003
JUVENILES	0.9233	0.0767	0.8720	0.1283	0.9113	0.0343	0.0492	0.0035	0.0012	0.0006
ADULTS	0.7863	0.2137	0.8350	0.1646	0.8806	0.0403	0.0714	0.0049	0.0017	0.0009
ALL 3 METHODS	**% AA**	**% AR**	***N* TOTAL**	***N* ADMIXED**	**Species contribution to Iceland:**			m_R	m_Y	
AA	0.991	0.009	548	5				0.926	0.966	
AR	0.025	0.975	199	5				0.074	0.034	
IC	0.892	0.108	259	28						

When assessing the parental contribution to the Icelandic populations by calculating the admixture proportion based on a coalescent approach and considering only gene frequencies, the contribution of AA in Iceland was high (92.6 %), while only 7.4 % of AR contributed to the gene pool in Iceland, When including molecular distance between alleles, the contribution of AR was lower (3.4%). The proportions of the six predefined hybrid classes were assessed by using the prior knowledge of both species as input. In Europe, 98.9 % was assigned to AA, 0.1 % to AR, 0.70% to the F_1 hybrid class, 0.08 % to the F_2 hybrid class and less than 0.2 % to backcrosses. In America, 96.5% was assigned to AR, 0.8 % to AA, 2 % to the F_1 hybrid class, 0.4 % to the F_2 hybrid class and less than 0.2 % to backcrosses (Table 3, Figure 5).

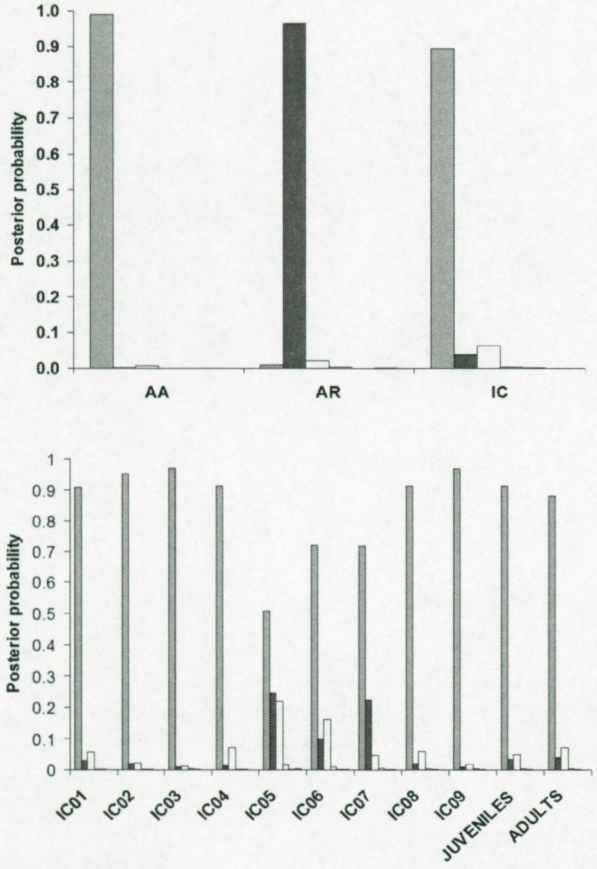


Figure 5: NEWHYBRIDS hybrid class probabilities of European, American and Icelandic *Anguilla* showing the proportion of pure AA (light grey), pure AR (dark grey), F_1 hybrids (white) in the pure species populations and in the Icelandic samples grouped (upper panel) or all Icelandic samples separately (lower panel). No F_2 hybrids, backcrosses with AA or backcrosses with AR could be detected.

A different pattern was observed in the Icelandic samples: in total 89 % of the individuals were pure AA, 3.8 % were pure AR and 6.3 % were F_1 (Table 3). No posterior probability was the highest for F_2 and backcross hybrid classes in any population. On average, there were more admixed individuals in adults than in juveniles. When testing the discriminative power of four moderately variable versus all seven loci, there was virtually no differentiation in outcome between both analyses (data not shown). When combining the results of all three assignment methods by selecting only admixed individuals detected by at least two techniques, an admixture proportion of 10.8 % American eel genomic material was detected within Icelandic populations, while only 1% and 2.5% of foreign genetic material was detected in the European and American eel, respectively (Table 3).

Relationship between meristic and genetic markers - A regression analysis between the mean number of vertebrae and the Bayesian admixture coefficient (q) yielded a significant positive correlation ($r = 0.46$; $p < 0.001$) (Figure 6). The regression improved even more when only admixed individuals ($q < 0.90$) were used for the analysis ($r = 0.58$ and $p < 0.001$) (Figure 6).

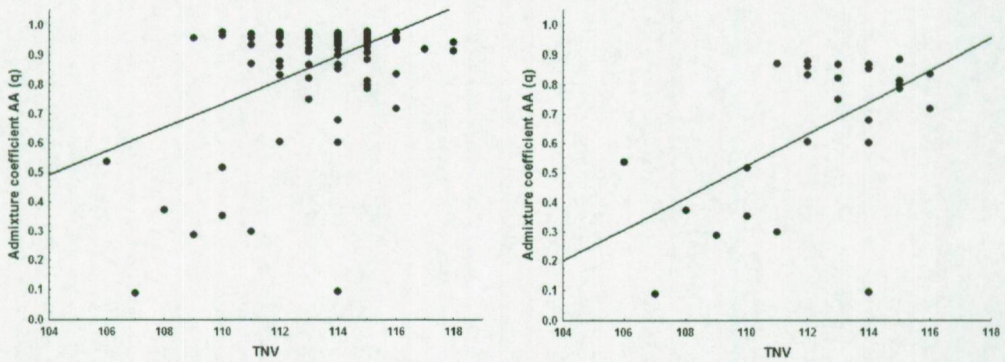


Figure 6: Correlation between the Total Number of vertebrae (TNV) and the admixture coefficient (q) of all Icelandic glass eels (left) and only Icelandic individuals exhibiting $q < 0.90$ (right).

DISCUSSION

Genetic diversity of North Atlantic Eels

Overall genetic variability estimates in European and American eel were similar to the values observed in other marine species with large effective population sizes (Ruzzante, 1998, Shaw, 1999; Roques *et al.*, 2001; Hutchinson *et al.* 2003; Knutsen *et al.* 2003) and correspond with previous microsatellite surveys in eel (Daemen *et al.*, 2001; Wirth and Bernatchez, 2001,

2003; Dannewitz *et al.*, 2005). The American eel shows a significantly higher gene diversity and allelic richness than the European eel, although no differences were found in observed and expected heterozygosities or polymorphism. Both species exhibit a similar life history with a long spawning migration and larval drift of almost a year, resulting in a high chance for dispersal, generation overlap and a large geographical distribution (Tesch, 2003). Nevertheless, the European eel is expected to maintain a larger effective population size, as it colonizes a much larger habitat and shows a higher age at maturity (Tesch, 2003). Additionally, previous studies showed more evidence for heterogeneity within the European eel compared to the American eel (Maes & Volckaert, 2002; Wirth & Bernatchez, 2003), a pattern expected to maintain genetic diversity in a species (Frankham, 2002). Accordingly, we only observed genetic heterogeneity among European locations but not among American locations. The slightly higher genetic diversity in the American eel may be attributable to the stronger decline in European eel recruitment in the last decades, partly due to the high fishing pressure and the numerous obstructions of the migration routes in Europe (Dekker, 2003). Alternatively, a lower historical effective population size within the European eel during glaciations has been suggested (Wirth & Bernatchez, 2003). Finally, the European eel might be a sister species of the American eel (Williams & Koehn, 1984; Avise *et al.*, 1990; Tsukamoto *et al.*, 2002), only retaining a subset of the original genetic diversity. In the larger scale study of Dannewitz *et al.* (2005), the American eel (N = 260) also showed a higher gene diversity and allelic richness than the European eel (N = 2626) at six out of eight loci, indicating that sample size are not the cause of our observation.

Genetic differentiation between the North Atlantic eels

As expected in marine species, the pattern of genetic differentiation within species was low ($F_{ST} < 0.01$) (Waples, 1998), and congruent with the hypothesis of panmixia in the American eel (Wirth & Bernatchez, 2003) and a subtle spatio-temporal heterogeneity within the European eel (Dannewitz *et al.*, 2005). Our study shows a high genetic differentiation between American and European eel, with multilocus pairwise differentiation (F_{ST}) values as high as 16.3 % and monolocus values of up to 69 % (AAN 03). Such high values contrast with earlier microsatellite surveys, where a maximal F_{ST} of 5% was observed (Mank & Avise, 2003; Wirth & Bernatchez, 2003). The discrepancies among studies are probably due to the fact that different sets of microsatellites were used. Our study included a combination of four moderately polymorphic and four highly polymorphic loci. Using the latter, F_{ST} values were

in the same range as detected in previous studies, which might be related to homoplasy (Mank & Avise, 2003). Estoup *et al.* (2002) recently showed that homoplasy is expected to reach the highest values when population sizes and mutation rates are high, characteristics that apply to marine fish and microsatellite loci, respectively. Species discrimination with moderately polymorphic markers is preferable (Manel *et al.*, 2002), as it lowers the probability to score Identical-In-State (IIS) alleles due to irregular mutation patterns or length restriction. The strategy of using only moderately variable SSR loci proved successful in the discrimination of various eel species (Maes *et al.*, 2005, in prep), where F_{ST} was negatively correlated to microsatellite polymorphism. The most discriminating loci between the American and the European eel (and contributing the most to the high F_{ST} observed) were the three lowly polymorphic loci (AAN 01, AAN 03, AAN 05). Locus AAN 05 was the most discriminating between the two species, indicating that less polymorphic loci exhibiting a lower mutation rate could better reflect long-term historical divergence (Estoup *et al.*, 2002).

Evidence for hybridisation in Icelandic samples

The Icelandic samples clearly exhibited patterns of mixed ancestry, detected by an intermediate level of genetic variability at three diversity estimators (heterozygosities, genetic diversity and allelic richness), intermediate allelic frequencies between American and European eel at loci AAN 01, AAN 03 and AAN 05, Hardy-Weinberg deviations, significant gametic disequilibria, and especially by a higher proportion of American genomes than in European populations (11-15% versus 1%). All methods (the classical and two Bayesian methods) yielded congruent results, suggesting that the Icelandic samples consist largely of European eel (89%), but with a high admixture (10.8%) of American eel material. By comparison, Avise *et al.* (1990) observed only 2-4% of American eel material in Icelandic samples. The more than double amount of admixture observed in our study might be attributed to the multiple polymorphic unlinked nuclear loci, which increases the power of hybrid detection (Boecklen & Howard, 1997; Anderson & Thompson, 2002). Our estimates of admixture within Iceland (10-15%, depending on the method used) were similar to other hybridizing marine taxa. (redfish: Roques *et al.*, 2001; flatfishes: She *et al.*, 1987). To discriminate between hybridisation and accidental recruitment of pure American eels in Iceland, admixture analyses enable the classification of genotypes as originating from one (pure) or two (hybrid) species. Icelandic genotypes consisted largely of parental species (89.3% AA and 3.8% AR), but strong evidence for the presence of admixed individuals with

common ancestry from both species (6.3 %) was also detected. A pure mechanical common distribution of both species is not concordant with such data.

The genetic identification of hybrids was confirmed by vertebral counts. While the number of vertebrae in American eels ranged from 104 to 110, 99.4% of European eels exhibited 111 vertebrae or more, with only 2 out of 296 European eels exhibiting 110 vertebrae. In agreement with microsatellite data, the number of vertebrae in Icelandic individuals showed an intermediate position (106-120 vertebrae) between the American and the European eel. A higher proportion of low vertebral count individuals (7.4 % with TNV < 110) than in Europe was observed. Furthermore, a strong positive correlation was observed between genetic admixture coefficients and the total number of vertebrae, which indicates that the basis of this trait is genetic and not environmental. Avise (1990) suggested that Icelandic individuals exhibiting less than 110 vertebrae either were pure *A. rostrata* expatriates migrating together with *A. anguilla* individuals, rare *A. anguilla* individuals showing environment-related ontogenic abnormalities or hybrids between the two species. The detection of F_1 hybrids besides pure American eel individuals in our study clearly supports the latter explanation. The second explanation is unlikely, as the strong correlation between the genetic admixture coefficient q and the total number of vertebrae constitutes evidence in favour of a genetically based number of vertebrae.

Our data can only be interpreted as evidence for a hybrid zone in the Sargasso Sea, still detectable at about 6,000 km from its origin. Interestingly, some admixed individuals were also detected in Morocco, in the southernmost distribution area. Hybrids are known to colonize and survive better in intermediate niches or challenging habitats (Dowling & Secor, 1997), the extremely disjunct distribution of admixed eels and their survival until adulthood (silver eels) may point to a similar pattern. The occurrence of admixed and absence of pure American eel in Morocco rules out an anthropogenic contribution to this pattern. In Iceland, the admixture proportion was similar across life stages, although we detected more American-like genomes in adults, which might point to temporal stability and a better survival of hybrids in marginal habitats. We have a common sampling site with the study of Avise *et al.* (1990) (Reykholar), where a higher proportion of hybrids and pure AR was also found (IC6 and IC7). This suggests either differential survival of hybrids in certain habitats or the importance of current patterns advecting American like larvae to Iceland (Avise *et al.*, 1990; Dowling & Secor, 1997). Within Icelandic samples exhibiting hybrids, an unequal contribution of the two species was detected, pointing to the genetic introgression of American eel material into European eel. While a small proportion of European eel material

was found in American eel samples (2.5 %), American eel material accounted for only 1% in Europe, but 11 % in Iceland.

A primary or secondary tension zone in the Sargasso Sea ?

The nature of hybrid zones in marine organisms remains difficult to define due to difficulties in assessing the selection forces acting on genotypes at sea and due to the sympatric reproduction of several species, blurring the signal of primary or secondary contact (Barton & Hewitt, 1995; Nielsen *et al.*, 2003). The low number of hybrid genotypes observed in this study may have two causes. First, both species spawn at different times and places, although partly in sympatry (McCleave, 1993;Tesch, 2003). One-week-old larvae are recorded in the Sargasso Sea with a huge distributional overlap, suggesting a similar overlap in spawning time/site in both species. No adult eel has ever been observed spawning, so other pre-zygotic barriers like spawning depth, assortative mating and gametic incompatibility are highly speculative and untestable at this time. The reinforcement of differential spawning location or behaviour, through reproductive character displacement is, however, a likely explanation (Servedio, 2004). Reinforcement is known to be strongest in sympatric zones; hence, hybrid avoidance might well be achieved through differences in spawning depth in the zone of overlap. Secondly, the distribution of genotypes in Icelandic samples fits a tension zone model, in which selection acts against hybrid individuals, which present a lower fitness in comparison with parental species. Icelandic genotypes were made up mostly by parental species (89.3 % AA and 3.8 % AR), while hybrids showed no superiority, with only occasional F_1 and no F_2 hybrids (6.3 %). In a tension zone, selection must act strongly on hybrids in order to lower their numbers (Barton & Hewitt, 1985). We detected a significant gametic phase disequilibrium in the Icelandic samples, which may be the consequence of genomic stress within hybrids, concordant with a tension zone. Further, no backcrosses were observed using the NewHybrids admixture approach (Anderson & Thompson, 2002), pointing either to a lower reproductive fitness of hybrids, to their very low number or to a lack of discrimination power using our set of markers. In the model based clustering method of Pritchard *et al.* (2000), however, we arbitrarily discriminated three genotype classes, albeit with high confidence interval. Only 78% was assigned with high confidence to the European eel, while 21 % showed the signal of admixture with one or the other species. Combining both results, we conclude that only the use many almost diagnostic markers may enable the detection of more complicated hybrid classes, such as backcrosses and F_2's (Anderson &

Thompson, 2002). Icelandic individuals have nevertheless an increased chance to reproduce with European eel, as they migrate with the same currents to the Sargasso Sea (Tsukamoto *et al.*, 2002; Tesch, 2003).

The low number of F_1 hybrids observed indicates that the hybrid zone between the American and the European eel is most likely a secondary tension zone, maintained by a balance between natural selection and extensive dispersal to safeguard species integrity. Both American and European eel share the same spawning grounds (the Sargasso Sea between 20-35°N), which provides an opportunity for hybridisation (Palumbi, 1994). In North Atlantic eels, spawning site migration seems to have evolved together with oceanic current shifts under the influence of glaciations (Tsukamoto *et al.*, 2002). After an initial sympatric speciation some 3-10 million years ago (Aoyama *et al.*, 2001; Lin *et al.*, 2001) under the influence of ice sheets inducing a disjunct distribution of eels in the North-Atlantic, assortative mating and spawning diachrony probably maintained reproductive isolation. Due to the unpredictability in current patterns combined with an increasing migration distance and duration after recolonisation, a secondary contact between the species may have arisen. In this sense, eels cover an above average distance (6,000 km) to reach the spawning grounds, which is likely to increase the chance for temporal overlap between species. On the other hand, a recent re-evaluation of sympatric speciation alleged that reproductive isolation mechanisms could arise with a significant amount of dispersal/gene flow between diverging species (Mallet, 2005). Given the active component of larval migration of both North-Atlantic species, reinforcement may be very strong to maintain the East-West migrational cue of larvae, with incidental hybridisation occurring.

Despite the peak spawning season being in February for the American eel and in April for the European eel, larval surveys indicate a large overlap in hatching larvae (7 mm) of American and European eel in the Sargasso Sea (McCleave *et al.*, 1993). The observation of a hybrid zone with a bimodal pattern of dominating parental genotypes and only occasional hybrids points to a combination of spatio-temporal disjunct spawning, pre-zygotic barriers (behavioural control increases assortative mating between conspecifics) and post-zygotic barriers (strong selection against hybrids).

Do Icelandic hybrid eels represent an evolutionary dead end ?

The paradigm of why hybrids are mainly found in Iceland remains. Although the North Atlantic Drift passes west of the British Isles, no hybrids were found in Ireland, which might

point to an active process of trans-Atlantic dispersal. Studies on birds show that intermediate migratory behaviour of hybrids is mostly maladaptive and decreases overall fitness (Berthold, 1988). This explanation holds for the Icelandic eels, possibly showing an active intermediate migration by bilateral genetic imprinting. Admixed individuals found elsewhere in Europe are defined as second-generation hybrids, most likely originating from backcrosses with Icelandic F_1 hybrids. Selection must thus act on pre-zygotic barriers and subsequent hybrids fitness to maintain the species integrity, doomed to be lost if intermediate genotypes survive equally well.

ACKNOWLEDGMENTS

Research was funded by the European project EELREP (Q5RS-2001-01836). Samples were kindly provided by B. Jonsson, R. Poole, J. Van Willigen, P. Elie, A. Crivelli, A. Yahyaoui, G. Wippelhauser (S. Zink) and J. Aoyama. Helpful comments on earlier versions were given by T. Wirth, E. Nielsen, P. Galbusera, L. De Meester and F. Ollevier. G.E. Maes was supported by an IWT (Institute for the Promotion of Innovation by Science and Technology in Flanders) PhD scholarship. J.M. Pujolar received a postdoctoral fellowship from the Ministerio de Educación, Cultura y Deporte (Spain).

PART B:

Spatio-temporal genetic structure in European eel (*Anguilla anguilla* L.)

CHAPTER III

Clinal genetic variation and isolation by distance in the European eel *Anguilla anguilla* (L.)

Maes G.E. and Volckaert F.A.M.

SUMMARY

The genetic variability and structure of the European eel (*Anguilla anguilla* L.) in populations throughout Europe was reassessed using 15 allozymic loci, 7 of which were polymorphic. Seven sites were sampled on a latitudinal gradient across the natural continental range, extending from southern France to southern Norway. Heterozygosity (H_e = 0.05) and level of polymorphism (P = 0.43) were comparable to other marine fish. Populations were poorly differentiated (G_{ST} = 0.014, F_{ST} = 0.002), which is not surprising considering the high dispersal capability of the European eel. However, a significant geographical cline was detected at two alleles (*IDH-1*100* and *GPI-1*110*), and genetic distances (D_{CE}) were concordant with geographical coastal distances. Mantel tests, pairwise F_{ST}'s and multidimensional scaling analyses identify three distinct groups: Northern Europe, Western Europe and the Mediterranean Sea. We propose that the clinal genetic structure in the European eel may be due to 1) isolation by distance (as recently detected with microsatellites), 2) temporal reproductive separation, 3) post-larval selective forces, 4) contact between formerly separated groups or 5) some combination thereof.

Keywords: allozymes; Atlantic Ocean; genetic variation; marine organisms; population genetics; selection; spatial structure

This chapter is published in: Biological Journal of the Linnean Society 77: 509-521.

INTRODUCTION

Marine fishes often maintain large effective populations, are highly fecund, and have a high potential for larval dispersal. There is thus considerable doubt as to whether natural marine populations maintain separate reproductive units and whether genetic approaches might be useful to discriminate the population structure (Waples, 1998). For example, in some marine taxa, information from allozymes and mitochrondrial DNA was unable to reveal obvious genetic subdivisions (Ward, Woodwark & Skibinski, 1994: Grant & Bowen, 1998) whereas in many coral reef fishes genetic structuring is evident (Waples, 1987, Planes, Doherty & Bernardi, 2001; Planes & Fauvelot, 2002). The marine environment, however, holds many peculiarities, which, in conjunction with other life-history characteristics of marine fishes, hints at genetic differentiation within apparent panmictic populations. In fact, the marine environment shows much more heterogeneity owing to the influence of climate, hydrodynamics and topography on natural barriers, which affects dispersal (Cowen *et al.*, 2000). Genetic structuring is enhanced furthermore by certain biological traits, such as sex-dependent migration, phylopatry and assortative mating, which can counteract dispersal and gene flow (Sinclair, 1988; Ruzzante, Taggart & Cook, 1998). Hence, numerous marine species maintain a genetic structure despite a great potential for dispersal (Shaw, Pierce & Boyle, 1999; Nielsen *et al.*, 2001).

Although the European eel (*Anguilla anguilla* L.) spends most of its lifetime in freshwater systems or estuaries, its early life-history is comparable to that of other marine organisms. From the Sargasso Sea breeding site, the leptocephali of the European eel move actively to the continental shelf of the eastern Atlantic seaboard (Arai, Otake & Tsukamoto, 2000), where they metamorphose. Thereafter, the glass eels may ascend to the rivers although this is not always the case (Tsukamoto, Nakai & Tesch, 1998). After several years spent in freshwater as feeding yellow eels, partially mature silver eels migrate back to their natal spawning grounds. Here they complete maturation, reproduce only once and die. Fecundity, larval mortality and dispersal potential are high (Tesch, 1977). It has been difficult to evaluate the presence of any population structure due to the paucity of available information concerning the life-cycle and genetic structure of the European eel (de Ligny & Pantelouris, 1973; Comparini & Rodino, 1980, Yahyaoui, Bruslé & Pasteur, 1983). It was long assumed that European eel behaved as a panmictic population, i.e. a homogeneous population spawning in the Sargasso Sea. Early studies, based on allozymes, suggested that European eel populations differ between several continental European locations (Drilhon *et al.*, 1967;

Pantelouris, Arnason & Tesch 1970). This conclusion, however, was rejected on methodological grounds (Koehn, 1972). Later, mitochondrial DNA studies failed to detect any genetic differentiation (Lintas, Hirano & Archer, 1998; Daemen *et al.*, 2001). Nevertheless, other indications, such as hybrids between European and American eel in Iceland (Avise *et al.*, 1990), separate populations in the northern and southern range (Daemen *et al.*, 2001) and isolation by distance using microsatellite markers (Wirth & Bernatchez, 2001) constitute elements challenging the panmixia hypothesis of European eel.

Clinal variation on a latitudinal gradient has been detected in *Anguilla rostrata*, the American eel, (Koehn & Williams, 1978) and *A. japonica*, the Japanese eel, (Chan *et al.*, 1997), prompting a re-evaluation of the allozyme data for *A. anguilla* following the isolation by distance hypothesis suggested by Wirth and Bernatchez (2001). The objective of this study was twofold: We first reassessed the genetic variability and structure of the European eel by using most of the allozyme loci screened in earlier studies on eels. We then tested whether there was clinal variation in the European eel similar to that detected in the American eel (Koehn & Williams, 1978) and the Japanese eel (Chan *et al.*, 1997). We sampled *A. anguilla* populations found on the North-western Atlantic and Mediterranean shelf to test for latitudinal variation and to determine whether the Mediterranean and Atlantic populations could be differentiated as proposed in earlier studies (Pantelouris, Arnason & Tesch 1970, Yahyaoui, Bruslé & Pasteur, 1983).

MATERIAL AND METHODS

Material -. We collected seven samples of adult eels, mainly during the months when silver eels start their migration back to the Sargasso Sea. Each sample consisted of approximately 50 individuals. Five of the samples were Atlantic coast representatives and two of the samples were Mediterranean Sea representatives (see Table 1 & Fig. 1 for specific details). Except for The Netherlands site where one all male and one all female sample were collected, all samples consisted of both males and females. The eels were collected and brought back to the laboratory alive, where they were processed.

Allozyme electrophoresis - Genotypes were detected by horizontal Starch Gel Electrophoresis (SGE) in the five Atlantic samples and Cellulose Acetate Gel Electrophoresis (CAGE) in the two Mediterranean samples (Harris & Hopkinson, 1976; Richardson, Baverstock & Adams,

1986), because of the rapidity and ease of the latter method. Liver and muscle tissue was homogenized with a double volume of 10 mM Tris-HCl (pH 7.8) at 4°C.

Table 1 : Location of *Anguilla anguilla* L. samples taken across Europe; *N* = number of samples; Y = yellow eel; S = silver eel; m = males; f = females.

Sampling station	Country	Code	Longitude & latitude	Sampling date	Life stage	*N*
Bergen	Norway (N)	NRW	60°24'N; 05°20'E	11/10/1997	Y + S	50
Mayo	Ireland (EI)	IRL	53°55'N; 09°55'W	23/09/1997	S	50
Den Oever	The Netherlands	NE1	53°01'N; 05°13'E	01/10/1997	S (m)	50 (m)
Den Oever	The Netherlands	NE2	52°27'N; 05°17' E	29/10/1997	S (f)	50 (f)
Pleurtuit	France (F)	FR1	48°34'N; 02°03'W	09/1997	Y + S	26
Pila	Italy (I)	ITA	44°54'N; 12°22'E	04/1999	Y + S	28
Sète	France (F)	FR2	43°24'N; 03°41'E	12/1998	Y + S	50

The samples were centrifuged for 10 min at 13,000 rpm (10,000 g) at 4°C and the supernatant was aliquoted and stored at - 80°C. We followed the procedures of Whitmore (1990), Pasteur *et al.* (1987) and Hoelzel (1992) for Starch Gel Electrophoresis (SGE) and the procedure of Richardson *et al.* (1986) for Cellulose Acetate Gel Electrophoresis (CAGE). Our staining procedures followed Hebert & Beaton (1989). We used Shaklee *et al.*'s (1989) nomenclature for enzymes. The two techniques for allozyme electrophoresis were calibrated to ensure interpretation was consistent and reliable. To detect scoring artefacts, we aligned all the alleles and repeatedly ran some loci for selected samples with both methods. Ultimately we analysed 9 enzymes (15 loci) on CAGE and SGE which could be genotyped after a few modifications. Seven polymorphic loci were included to assess population structure (Appendix 1 and Table 2).

Table 2 : *Anguilla anguilla* L. : Enzymes scored in tissue (liver and muscle) extracts and buffers used in the electrophoresic analysis. TLCB = Tris-Lithium-Citrate-Borate, TC = Tris-Citrate, P = Poulik, TM = Tris-Maleate and TG = Tris-Glycine. The seven polymorphic loci included in the analysis are shown in bold. Nomenclature for enzymes as in Shaklee *et al.* (1989).

Enzyme	E. C. number	Tissue	Gel buffer system	Locus	Nr of alleles
Aspartate aminotransferase	2.6.1.1	Liver	TLCB, TM	***AAT-1****	7
Glucose-6-phosphate isomerase	5.3.1.9	Liver	TLCB, TG	***GPI-1****	4
Phospho-glucomutase	2.7.5.1	Muscle	TLCB, TG	***PGM-1****	2
L-Iditol dehydrogenase	1.1.1.14	Liver	TLCB, TG	*IDDH-1**	1
Malic enzyme	1.1.1.40	Liver	TLCB, TM	*MEP-1**	1
Malate dehydrogenase	1.1.1.37	Liver	TLCB, TM	***MDH-1****	2
L-lactate dehydrogenase	1.1.1.27	Muscle	P, TM	*LDH-A**	1
Isocitrate dehydrogenase	1.1.1.42	Liver	TC, TM	***IDH-1****	7
Fumarate hydratase	4.2.1.2	Liver	TC, TM	*FH-1**	1

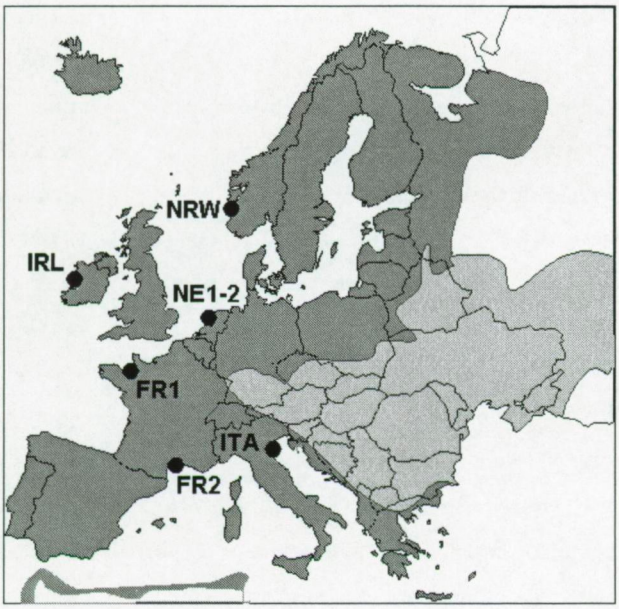


Figure 1 : Sampling locations (sampling codes) and presumed natural range (dark area) of European eel (*A. anguilla* L.). NRW : Norway (Bergen); IRL : Ireland (Burrishoole); NE1 & NE2 : The Netherlands (IJsselmeer); FR1 : France (Pleurtuit); ITA : Italy (Po); FR2 : France (Sète).

Data analysis of genotypes - Genetic diversity was evaluated based on genotype and allele frequencies, the level of polymorphism ($P_{(0.99)}$ criterion, where a locus is considered polymorphic when the frequency of the most common allele does not exceed 0.99), observed and expected heterozygozity (H_o and H_e), number of alleles and mean number of alleles per locus (MNA). Homogeneity of allele frequencies among samples was tested with GENEPOP version 3.1d (Raymond & Rousset, 1995). Departures from Hardy-Weinberg equilibrium were calculated as $D = (H_o - H_e) / H_e$ with GENEPOP version 3.1d (Raymond & Rousset, 1995) using the Markov chain method. The standard deviation of each value was estimated by jack knifing over loci as implemented in GENETIX version 4.02 (Belkhir *et al.,* 1999) and the linkage disequilibrium was calculated using the LINKDIS procedure implemented in GENETIX (Belkhir *et al.,* 1999). Population structure was characterized using a G-test of differentiation (Raymond & Rousset, 1995), hierarchical F-statistics, theta (θ) and G_{ST}-values as implemented in the GENETIX 4.02 software package (Belkhir *et al.,* 1999). Because of the subtle differentiation and the large number of rare alleles, we chose to estimate the fixation index (F_{ST}(RB)) following Robertson & Hill (1984) after correction by Raufaste &

Bonhomme (2000), but used Weir and Cockerham's theta estimator for highly differentiating loci (when F_{ST} >0.05). Significance of multilocus F_{ST} was assessed with permutation tests (1000 replicates), which yielded a distribution of F_{ST} under the null hypothesis of no significant population differentiation, followed by a comparison with the observed F_{ST} value. Pairwise genetic distances (D_{CE}) were calculated according to Cavalli-Sforza & Edwards (1967), considering drift as the only force acting on genetic variability with fluctuating effective population size. The significance level of the genetic distances was obtained by permuting (1000 permutations) individuals between samples for each pair of samples being compared (GENETIX). The correlation between genetic distances (D_{CE}) and geographical distances, measured either as the shortest coastal distance between two samples or the distance between the sampling site and the spawning grounds (26° 54' N; 51° 03' W), was performed using Mantel's non-parametric test on pairwise distance matrices (Mantel, 1967) using the MANTEL procedure in GENETIX (Belkhir *et al.*, 1999). A correlation test was performed between allele frequencies and latitude to test for clinal variation (STATISTICA 5.0, Statsoft). In all cases significance levels were corrected for multiple comparisons using a sequential Bonferroni correction (Rice, 1989). Finally, multi-dimensional scaling (MDS) of pairwise F_{ST}'s (with 100 iterations) was performed to project genetic differentiation between samples on a two-dimensional plane as implemented in the software package STATISTICA version 5.0 (Statsoft).

RESULTS

Genetic diversity - Seven of the 15 loci were polymorphic ($P_{(0.99)}$ = 47%). The total number of alleles per locus ranged from 1 to 7 and from 1.7 to 2.1 per sample per locus. Various rare and several private alleles were detected (Appendix 1). The average heterozygosity across all samples and loci was 0.05 (Table 3). Observed and expected heterozygosities per sample ranged from 0.05 to 0.06. Loci *GPI-1*, MDH-2*, AAT-1** and *IDH-1** showed the highest level of polymorphism, with expected heterozygosities of 0.27, 0.21, 0.19 and 0.08, respectively. *IDH-1** and *AAT-1** had the highest number of alleles (7), with several private alleles in the former and two null alleles in the latter one. The other polymorphic loci (*PGM*-1, AAT-2** and *MDH-1**) were rarely variable (observed heterozygosity between 0.005 and 0.020). Loci *IDH-2*, FH-1*, FH-2*, LDH-A*, LDH-B*, MEP-1*, MEP-2** and *IDDH-1** were monomorphic (Table 2). There was a significant decrease in the level of

polymorphism at higher latitudes ($r = -0.83$, $p < 0.05$) ranging from 40 % (Italy) to 26,7 % (Norway) (Table 3).

Departures from Hardy-Weinberg and gametic disequilibrium - All seven samples were in Hardy-Weinberg equilibrium supporting Mendelian inheritance of genotypes, confirming the quality of the genotype interpretation and the random association of alleles within samples. Averaged over all samples, the inbreeding coefficients of all loci did not differ significantly from zero (Table 3). The loci *AAT-2**, *GPI-1** and *MDH-2** were in linkage disequilibrium among each other and with the *IDH-1** locus in several samples (data not shown).

Table 3: Genetic variability estimates of *Anguilla anguilla* L. at 15 loci in 7 populations: average number of alleles per locus (MNA); percentage of polymorphic loci (0.99 criterion); observed and expected (non biased) heterozygosity (H_o and H_e, means $\pm$ SD) and multilocus F_{IS} estimates. * = $p < 0.05$, ^{NS} = non significant. For population abbreviation see Table 1.

Population	MNA	$P_{0.99}$	H_o	H_e (n.b.)	F_{IS} (*f*)
NRW	1.7	26.7	0.057 + 0.11	0.054 $\pm$ 0.11	-0.059 ^{NS}
IRL	2.1	26.7	0.049 $\pm$ 0.09	0.046 $\pm$ 0.08	-0.062 ^{NS}
NE2	2.0	33.3	0.047 $\pm$ 0.08	0.050 $\pm$ 0.08	0.068 ^{NS}
FR1	1.7	33.3	0.059 $\pm$ 0.10	0.057 $\pm$ 0.10	-0.029 ^{NS}
FR2	1.9	33.3	0.057 $\pm$ 0.10	0.061 $\pm$ 0.11	0.074 ^{NS}
ITA	1.8	40.0	0.052 $\pm$ 0.09	0.049 $\pm$ 0.09	-0.061 ^{NS}
All populations	2.4	46.7	0.052 $\pm$ 0.09	0.052 $\pm$ 0.003	

Multi-locus analysis of genetic structure - An overall probability test of genotypic differentiation at all loci and all samples based on the Markov Chain method was significant (G-test, $p = 0.04$). Overall genetic differentiation was low ($G_{ST} = 0.014$ and F_{ST} (RB) = 0.002) and non significant ($p > 0.05$ after a permutation test on 1000 replica's). The principal contributing loci to the genetic structure are in order of magnitude: *GPI-1**, *PGM-1**, *IDH-1**, *MDH-1** and *MDH-2** (Table 4). Pairwise F_{ST} over all loci was highly significant between the most distant samples, namely Italy and Norway (F_{ST} (RB) = 0.051, $p < 0.01$ after Bonferroni correction, Table 5). A test of genetic differentiation between each sample pair per locus showed a significant difference between all Atlantic samples and the Norwegian sample (NRW) at locus *IDH-1** (maximum F_{ST} (θ) = 0.05, $p < 0.01$), as well as between the Mediterranean and Atlantic samples at loci *MDH-2**, *IDH-1** and *GPI-1** (data not shown). Pairwise genetic distances (Cavalli-Sforza & Edwards, 1967) between all samples are generally small but all significant ($p < 0.01$). Nevertheless, the value between Norway (NRW)

and Italy (ITA) is the only significant one after Bonferroni correction for multiple tests (21 tests).

Table 4 : Inbreeding coefficient (F_{IS}), global inbreeding coefficient (F_{IT}), fixation index (F_{ST}) and level of gene flow (Nm) at each polymorphic locus. * = $p < 0.05$

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
*IDH-1**	-0.0376	-0.0274	0.0098	25.28
*GPI-1**	-0.0116	0.0001	0.0115*	21.50
*AAT-1**	-0.0154	-0.0094	0.0059	41.95
*AAT-2**	-0.0176	-0.0102	0.0073	34.03
*MDH-1**	-0.0182	-0.0026	0.0153	16.04
*MDH-2**	-0.0250	-0.0112	0.0134	18.40
*PGM**	-0.0194	-0.0054	0.0137	18.05
Multilocus	-0.0190	-0.0084	0.0104	23.73

The Norwegian and Italian samples exhibit the highest genetic distances between each other (D_{CE} = 0.045); the Italian sample is the most divergent from all other samples. Both Mediterranean samples cluster together, which is concordant with the F_{ST}(RB) values (Table 5).

Table 5 : Pairwise $F_{ST\,(RB)}$ estimates (θ, above diagonal) between European eel populations calculated following the corrected Raufaste & Bonhomme (2000) estimator. Genetic distances (D_{CE}, below diagonal) were calculated following Cavalli-Sforza & Edwards (1967). * = $p < 0.01$, ** = $p < 0.001$, § = significant after Bonferroni correction.

	$F_{ST}(\theta)$						
D_{CE}	NE1	NRW	IRL	NE2	FR1	FR2	ITA
NE1	0	0.0045	-0.0002	-0.0002	-0.0006	0.0029	0.0180
NRW	0.014*	0	0.0072	0.0030	0.0156	0.0070	0.0512§
IRL	0.008*	0.016*	0	-0.0000	0.0006	0.0064	0.0092
NE2	0.013*	0.010*	0.015*	0	-0.0022	0.0050	0.0203
FR1	0.010*	0.013*	0.013*	0.010*	0	0.0045	0.0223
FR2	0.013*	0.017**	0.016*	0.014*	0.010*	0	0.0201
ITA	0.029**	0.045§	0.025*	0.037**	0.043**	0.032**	0

A Mantel test was conducted with two genetic estimators (F_{ST} and Cavalli-Sforza chord distance (D_{CE})) and two geographical distances, namely the coastal distances between sampling sites and the difference in distance between sampling and spawning site (Sargasso Sea). A high correlation coefficient was found in the four cases, but the correlation was

slightly higher when calculated with the coastal distance among localities ($0.68 < r < 0.78$, $p < 0.05$) than with distance to the spawning ground ($0.63 < r < 0.80$, $p = 0.064$ and 0.050 respectively) (Fig. 2). The Italian sample is the main contributor to the pattern of isolation by distance as expected from the high differentiation estimators between this population and the others. When loci showing clinal variation are removed, correlation remains constant (*IDH-1** excluded) or increases in significance (*GPI-1** excluded). Locus *MDH-2** is the main contributor to the observed correlation, as when removed the p-value increases above significance level ($p > 0.1$).

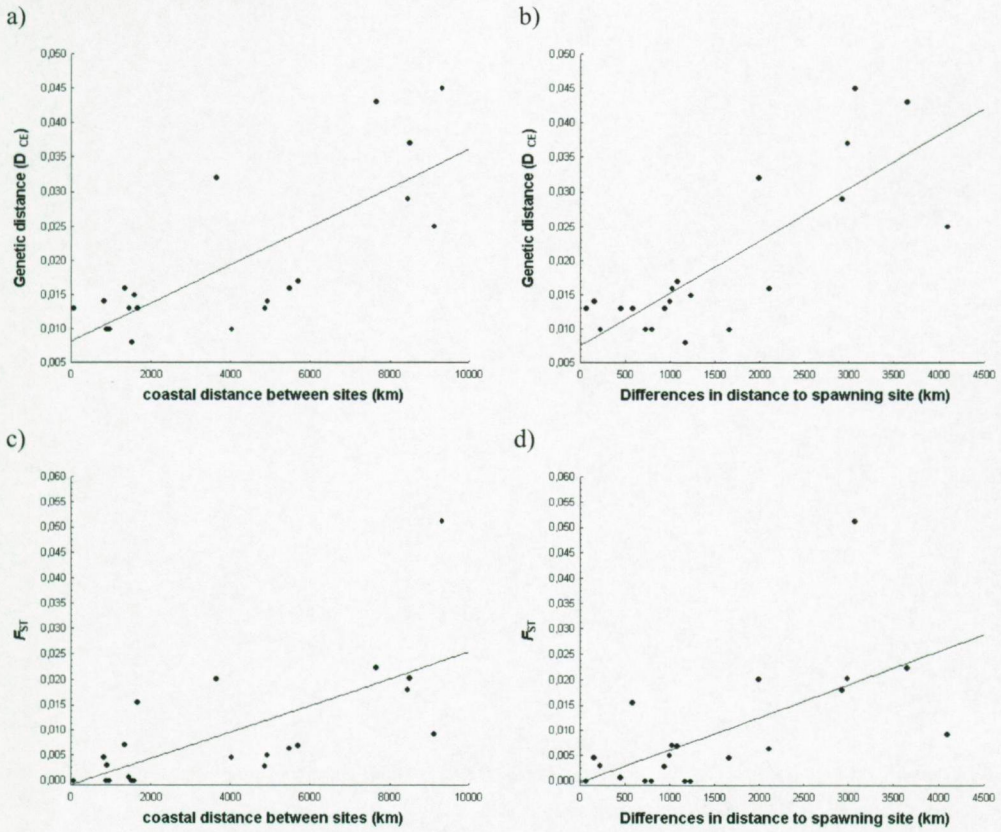


Figure 2 : Test for isolation by distance in European eel. Genetic distance (D_{CE}) based on seven polymorphic allozyme loci, (a) versus coastal geographical distances ($r = 0.78$, $p = 0.033$) and (b) versus distance from spawning site to sampling site ($r = 0.80$, $p = 0.050$). Genetic differentiation (F_{ST}) based on seven polymorphic allozyme loci, (c) versus coastal geographical distances ($r = 0.68$, $p = 0.013$) and (d) versus distance from spawning site to sampling site ($r = 0.63$, $p = 0.064$). Each point represents one of the 21 possible pairwise comparisons among seven samples. Pearson's correlation coefficient r and p-values result from Mantel's (1967) correlation test for dependent data.

Regression analysis between allele frequencies and latitude showed a clinal variation at two loci: Allele *IDH-1*100* showed a significant unidirectional increase with latitude ($r = 0.83$, $p = 0.02$), while allele *GPI-1*110* decreased with latitude ($r = -0.73$, $p = 0.06$) (Fig. 3a,b). When both samples from the Mediterranean were excluded from the dataset, the correlation became higher at both loci ($r = -0.99$ for *GPI-1** and $r = 0.98$ for *IDH-1**), more significant ($p < 0.01$) and the confidence interval was considerably narrower (Fig. 3c,d).

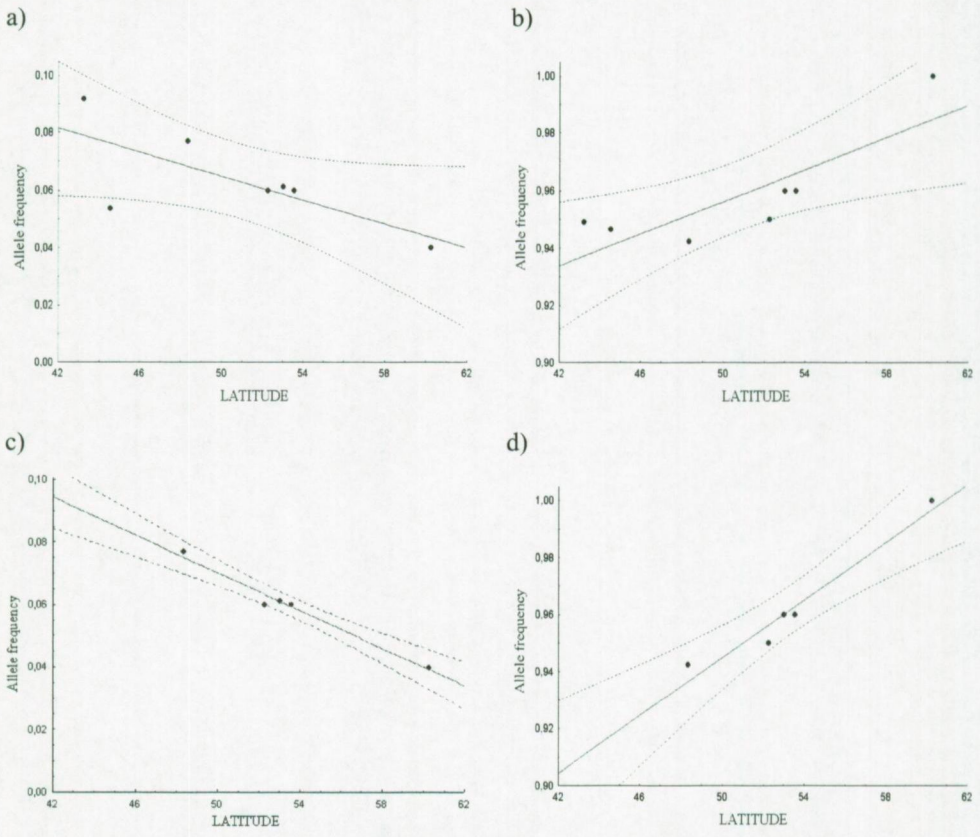


Figure 3 : *Anguilla anguilla* L. : Correlation between latitude (°N) and allele frequencies of (a,c) allele *GPI-1*110* and (b,d) allele *IDH-1*100*. Scatterplots (c) and (d) show the same correlation but excluding Mediterranean samples. The full line represents the regression, while the broken line indicates the 95 % confidence interval.

We conducted an MDS analysis on pairwise F_{ST} values between all samples (Fig. 4). The stress value was very low (< 0.005) when two dimensions were used; the relation between samples can thus be projected in a two-dimensional plane with high confidence. From the figure, it is clear that differentiation exists between the most distant samples (NRW and ITA)

and that the remaining samples are arranged according to an isolation by distance model. Indeed, if projected on the first dimension axis, all samples are roughly separated according to geographical distance between sites.

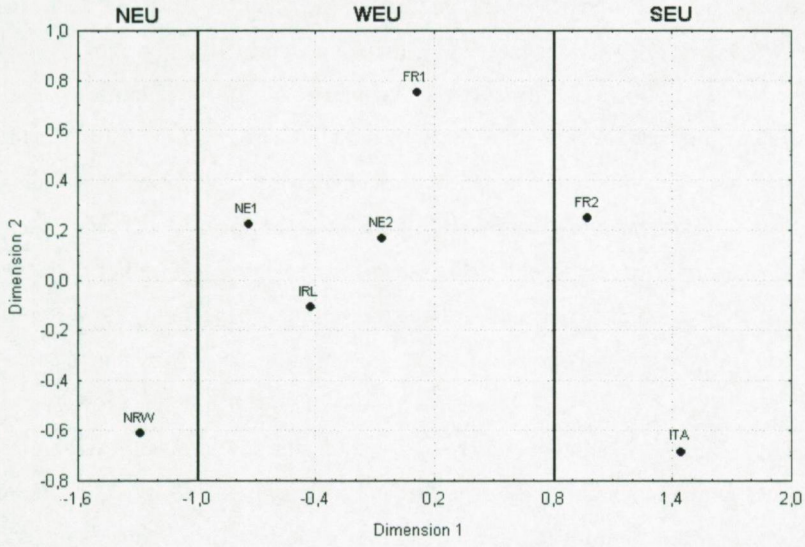


Figure 4 : Multidimensional scaling plot of pairwise F_{ST}'s between seven European eel populations. Stress value = 0.002. The first axis separates all populations according to geographical distance between sites, namely Northern (NEU), Western (WEU) and Southern (SEU) Europe.

DISCUSSION

Highly variable DNA markers enable scientists to reveal the subtle structure in seemingly panmictic populations. Several studies on marine organisms, with or without a dispersing larval phase, have demonstrated that such small differentiations are detectable using microsatellite DNA (Shaw *et al.*, 1999; Nielsen *et al.*, 2001). Nonetheless, allozymes are still frequently used for assessment of genetic variability in natural populations because large datasets are available, independent loci are numerous and the relative cost is low. The signal expected from allozymes has been suggested to be much lower than nuclear or mitochondrial DNA, because a lower level of polymorphism, the slower evolutionary rate of coding enzymes and selective constraints (Pogson *et al.*, 1995; Powers *et al.*, 1991). Nevertheless, the allozymic differentiation unveiled in highly vagile and supposedly panmictic populations (Kotoulas, Bonhomme & Borsa, 1995; Jerry, 1997), demonstrates that genetic structuring is

detectable at the level of allozymes. Furthermore, this structuring is concordant between several classes of molecular markers (Allendorf & Seeb, 2000).

The high effective population size (N_e = 0.5. 10^6; Daemen *et al.*, 2001), the high level of enzymatic polymorphism ($P_{(0.99)}$ = 47 %, this study) and the low genetic variability (H_e = 0.05, this study) of the freshwater eel are for the most part comparable with other marine teleosts (Ward *et al.*, 1994; Bohonak, 1999), including several other species of eel (Williams, Koehn & Mitton, 1973; Chan *et al.*, 1997; Daemen *et al.*, 2001). The presence of rare and private alleles in our study is concordant with the high haplotype diversity and the star-like haplotype pattern encountered in other studies on European eel using mitochondrial DNA (Lintas *et al.*, 1998; Daemen *et al.*, 2001). It might be attributed to the high fecundity and non-random dispersal of leptocephali with occasional genetic sweepstakes during population expansions, similar to anchovies and sardines (see Grant & Bowen (1998) for an overview). The pattern of increasing polymorphism at decreasing latitudes shown here, is consistent with varying heterozygosity at the loci *IDH-1** and *AAT-1** (Table 3). We found that the heterozygosity of *IDH-1** gradually increased with decreasing latitude, resulting in the clinal variation of the most common allele (see further), whereas *AAT-1** was much more variable in the Mediterranean samples than in the Atlantic samples (10%). This result is consistent with the pattern observed in several aquatic organisms with recent habitat expansion where variability decreases with latitude (Bernatchez & Wilson, 1998; Bucklin & Wiebe, 1998).

Similar latitudinal differentiation was reported for the Japanese and American eels. Williams *et al.* (1973) found clinal genetic differentiation (up to 10 % difference in allele frequency) in the American eel, *A. rostrata*, along the eastern coast of North America. Later, Koehn & Williams (1978) attributed temporally stable latitudinal clines on the GPI and IDDH enzymes to natural selection. Chan *et al.* (1997) also found clinal genetic differentiation of 13 % and 9 % at two loci in the Japanese eel, *A. japonica*, along the Japanese coast. They proposed that the temporal differentiation (arrival time) and spatial distribution of young Japanese eels can account for the clinal variation. In the present study, we detected significant clinal variation of a comparable magnitude (namely 8 % from 43 °N to 60° N) at two loci (*IDH-1** and *GPI-1**). Earlier observations of a spatio-temporal allometric gradient in glass eel size and age at metamorphosis in silver eels strengthen the idea that European eel populations are distinct (McCleave *et al.*, 1998; Vøllestadt, 1992). Moreover, arrival waves have been monitored based on the condition index and meristic traits, possibly pointing to the presence of three distinct glass eel groups (Boëtius & Boëtius, 1989).

Our assessment of genetic variability and allozymic clinal variation shows a subtle differentiation between geographically distant samples (on a north-south axis), and more specifically between the Atlantic and Mediterranean oceanic environments (1-5 % F_{ST}). The strongest differentiation values were observed between the Norwegian and the Italian samples, which are also geographically the most distant. The Italian sample contributed the most to this differentiation, whereas the sample of Southern France (Sète) was intermediate between the Atlantic samples and the Italian sample (see MDS). There is a considerable ecological difference between the Western (Southern France) and the Eastern (Adriatic-Italy) Mediterranean basins (Margalef, 1985). Daemen *et al.* (2001) also found significant differentiation between a Moroccan sample and the remaining West-European samples using microsatellite DNA, suggesting that populations in the southern range are discrete.

Gene flow counteracts differentiation among populations caused by genetic drift or differential selection. When the distribution of a species is more or less continuous across its range, the balance between these antagonistic forces may result in clines. In this case, isolation by distance ensues: genetic differentiation at neutral loci increases with geographical distance. The life cycle of *A. anguilla* should facilitate gene flow. Other factors within the Sargasso Sea and The Gulf Stream, however, (e.g., physical barriers to pelagic stage dispersal, temperature and salinity fronts, homing behaviour of spawners, eddies favouring larval retention, differential post larval mortality during migration, and freshwater residency) may favour geographical isolation instead (Sinclair, 1988; Avise, 2004). Our results show a low but significant amount of differentiation between distant samples, suggesting a limited amount of gene flow between spawning populations. Furthermore, coastal geographical distance and genetic differentiation estimators (F_{ST} and D_{CE}) were significantly correlated, a finding similar to that of Wirth & Bernatchez (2001). Their study, based on microsatellite DNA, suggested an isolation by distance (IBD) or time scenario in the Sargasso Sea, which remained detectable along the European coast. Finally, Daemen *et al.* (2001) showed a cline in mitochondrial haplotype diversity, reinforcing the possibility of discrete populations across Europe. More subtle mechanisms separating eel populations must be active to explain the observed pattern described.

The findings from this study in conjunction with findings from previous studies (Harding, 1985; McCleave, 1993, Wirth & Bernatchez, 2001; Daemen *et al.*, 2001) provide new elements that can refine the current hypotheses that attribute clinal genetic variation and differentiation to spatio-temporal differentiation and selection.

The first classical hypothesis states that spatial differentiation contributes to genetic structuring at the spawning grounds: Maturing adults may spawn within their sub-population in the frontal zone (McCleave, 1993). This separation may be retained within the Gulf Stream by larval retention (Sinclair, 1988). The vastness of the Sargasso Sea (5.2 million km^2) and its heterogeneous hydrographical structure potentially limits contact between spawning groups, supporting the IBD scenario detected at both microsatellite and allozymatic markers (Wirth & Bernatchez, 2001; this study).

A second and likely scenario suggested by Chan *et al.* (1997) is that groups of spawning adults originating from different regions are temporally isolated and that this persists from year to year. Adult European eels begin their 6000 km long journey to the spawning grounds between September and December (Desaunay & Guérault, 1997) and arrive 6 months later. Data from fisheries indicate different departure times for different populations of European eels, which results in separate spawning groups in the Sargasso Sea. Hence, the groups are able to maintain their integrity throughout the arrival waves (Ruzzante *et al.*, 1998). The larval retention model combines temporal and spatial elements. Offspring fitness increases when individuals stay in the spatio-temporal proximity of their own "clutch". Thus offspring remain "members" of their natal population (member-vagrant hypothesis (Sinclair, 1988)). Unlike Sinclair, who claims the European eel belongs to one retention zone in the Sargasso Sea and, hence, constitutes one panmictic population, we argue that several temporally separated spawning units linked to a single (or multiple) retention zone(s) must be taken into account during spawning period. Unfortunately, studies exploring oceanic features to explain variance in the genetic composition of fish stocks remain scarce (Ruzzante *et al.*, 1998; Grant & Bowen, 1998; Stepien, 1999; Muss *et al.*, 2001).

Gene flow is the most probable cause for similarity between European eel populations. We thus propose that the larval retention model (member-vagrant hypothesis) with some degree of exchange between neighbouring populations (metapopulation) provides a realistic hypothesis to explain the present and previous results on eel population genetic structure (Sinclair, 1988; McQuinn, 1997). Vagrant individuals are considered important in maintaining the populations during historical expansion-contraction events, as fluctuations in reproductive success and purifying selection during migration could weaken and even eradicate certain populations. Hence immigrating individuals could account for the temporal persistence of the entire population (and its subpopulations) (Stepien, 1999).

The third hypothesis states that selection is the cause of the observed clinal variation at the loci *GPI-1** and *IDH-1**. If the evolutionary processes were strictly neutral, all loci

screened in this study should have been influenced simultaneously. Purifying selection and the wide dispersal capability of European eels (from 25° to 70° N) might have a selective impact on enzymes that are essential for respiration pathways (Horton *et al.*, 1996), like GPI and the temperature sensitive IDH (Sokolova & Portner, 2001). Allozymic clinal variation and differentiation has frequently been correlated with environmental factors in fish and shellfish taxa (Powers *et al.*, 1991; Gardner & Palmer, 1998) and in model organisms such as *Drosophila* spp. ('t Land *et al.*, 2000). Further investigations are needed to confirm this hypothesis.

Finally, genetic clinal variation may also be attributed to contact between previously isolated and genetically divergent populations. If subtle separate spawning sites with limited gene flow exist in the Sargasso Sea, mixing of the offspring during migration in the Gulf Stream (present pattern) could explain the observed clines at morphometric, allozymic, microsatellite and mitochondrial DNA markers (Harding, 1985; McCleave, 1993, Wirth & Bernatchez, 2001; Daemen *et al.*, 2001 and this study). An increasing overlap in spawning sites during past generations would also result in this pattern (historical pattern).

CONCLUSION

The evidence of clinal variation at several allozymes in the European eel presented in this study dramatically shifts the debate from whether population structure exists in the European eel to where and how the structure arises, and suggests that structure originates from an isolation by distance (or time) scenario, contact between formerly separated groups, or selection in a heterogeneous environment. We observed an increase in genetic distance with geographical distance along most of the distribution range of European eel suggesting a possible reproductive stock subdivision of this species. Our results are consistent with conclusions drawn based on microsatellite DNA in recent studies (Daemen *et al.*, 2001, Wirth & Bernatchez, 2001). Hence, allozymes remain useful to enable comparisons between several markers and generate additional knowledge about the complicated life history of North Atlantic eels. Further research in a spatio-temporal and life history context combining several markers (see Waples, 1998) is needed to improve stock structure assessment and to make optimal management decisions for a fishery suffering from a considerable decrease in yield since two decennia (Dekker, 2000).

ACKNOWLEDGMENTS

Research was funded by the Belgian Office of Scientific, Technical and Cultural Affairs (DWTC project MN/DD/42). Samples were kindly provided by F. Argenton, W. Dekker, E. Feunteun, G. Naevdahl and R. Poole. Helpful comments on earlier versions were given by L. Bernatchez, C. Pampoulie, C. Johnson, E. Gysels and one anonymous reviewer. The first author was supported by an IWT (Institute for the Promotion of Innovation by Science and Technology in Flanders) PhD scholarship during the preparation of the manuscript.

Appendix 1 : Allele frequencies at 7 polymorphic allozyme loci in 7 populations of *Anguilla anguilla*. H_e = expected heterozygosity, H_o = observed heterozygosity. For population abbreviations see Table 1.

	NE1	NRW	IRL	NE2	FR1	FR2	ITA	Total
*IDH-1**								
(N)	50	50	50	50	26	49	28	303
70	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.001
75	0.000	0.000	0.000	0.020	0.019	0.020	0.018	0.011
80	0.010	0.000	0.010	0.000	0.000	0.000	0.036	0.008
90	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.001
100	0.960	1.000	0.960	0.950	0.942	0.949	0.946	0.958
110	0.030	0.000	0.010	0.020	0.039	0.020	0.000	0.017
120	0.000	0.000	0.000	0.010	0.000	0.010	0.000	0.003
H_e	0.077	0.000	0.078	0.097	0.110	0.099	0.103	0.077
H_o	0.080	0.000	0.080	0.100	0.115	0.102	0.107	0.079
*GPI-1**								
(N)	49	50	50	50	26	49	28	302
80	0.010	0.000	0.020	0.000	0.000	0.010	0.071	0.016
90	0.061	0.130	0.040	0.080	0.077	0.102	0.000	0.070
100	0.867	0.830	0.880	0.860	0.846	0.796	0.875	0.851
110	0.061	0.040	0.060	0.060	0.077	0.092	0.054	0.063
H_e	0.240	0.293	0.220	0.250	0.272	0.348	0.226	0.269
H_o	0.224	0.300	0.240	0.240	0.269	0.347	0.250	0.268
*AAT-1**								
(N)	50	50	50	50	26	49	28	303
33	0.000	0.020	0.010	0.010	0.019	0.010	0.000	0.010
60	0.020	0.000	0.030	0.000	0.039	0.031	0.036	0.022
80	0.050	0.050	0.030	0.020	0.039	0.061	0.054	0.043
90	0.000	0.020	0.010	0.020	0.000	0.000	0.018	0.010
100	0.910	0.900	0.900	0.930	0.904	0.878	0.857	0.897
110	0.010	0.010	0.020	0.010	0.000	0.020	0.036	0.015
133	0.010	0.000	0.000	0.010	0.000	0.000	0.000	0.003
H_e	0.169	0.187	0.188	0.134	0.180	0.225	0.260	0.189
H_o	0.160	0.200	0.200	0.140	0.192	0.184	0.286	0.188
*AAT-2**								
(N)	50	50	50	50	26	50	28	304
90	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.001
100	1.000	0.980	0.990	0.990	0.981	0.980	1.000	0.989
110	0.000	0.020	0.000	0.010	0.019	0.020	0.000	0.010
H_e	0.000	0.039	0.020	0.020	0.038	0.039	0.000	0.023
H_o	0.000	0.040	0.020	0.020	0.038	0.040	0.000	0.023

	NE1	NRW	IRL	NE2	FR1	FR2	ITA	Total
*MDH-1**								
(N)	50	50	50	50	26	50	28	304
100	1.000	1.000	1.000	1.000	1.000	1.000	0.982	0.997
110	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.003
H_e	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.003
H_o	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.003
*MDH-2**								
(N)	50	50	50	50	26	50	28	304
70	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.003
80	0.040	0.050	0.070	0.060	0.077	0.040	0.000	0.048
90	0.040	0.050	0.010	0.020	0.019	0.070	0.018	0.032
100	0.870	0.840	0.900	0.890	0.865	0.890	0.964	0.889
110	0.050	0.060	0.020	0.030	0.039	0.000	0.000	0.028
H_e	0.237	0.286	0.185	0.203	0.243	0.201	0.070	0.213
H_o	0.260	0.320	0.200	0.160	0.269	0.180	0.071	0.214
*PGM-1**								
(N)	50	50	50	50	26	50	28	304
90	0.000	0.000	0.000	0.020	0.000	0.000	0.018	0.005
100	1.000	1.000	1.000	0.980	1.000	1.000	0.982	0.995
H_e	0.000	0.000	0.000	0.039	0.000	0.000	0.035	0.010
H_o	0.000	0.000	0.000	0.040	0.000	0.000	0.036	0.010

CHAPTER IV:

Panmixia in the European eel: a matter of time...

Dannewitz J.[‡], Maes G.E.[‡], Johansson L., Wickström H., Volckaert F.A.M. and Järvi T.

SUMMARY:

The European eel (*Anguilla anguilla* L.) has been a prime example of the panmixia paradigm because of its extraordinary adaptation to the North Atlantic gyral system, semelparous spawning in the Sargasso Sea and long trans-oceanic migration. Recently, this view was challenged by the suggestion of a genetic structure characterised by an isolation-by-distance (IBD) pattern. This is only likely if spawning subpopulations are spatially and/or temporally separated, followed by non-random larval dispersal. A limitation of previous genetic work on eels is the lack of replication over time to test for temporal stability of genetic structure. Here we hypothesize that temporal genetic variation plays a significant role in explaining the spatial structure reported earlier for this species. We tested this by increasing the texture of geographical sampling and by including temporal replicates. Overall genetic differentiation among samples was low, highly significant and comparable to earlier studies ($F_{ST} = 0.0014$; $p < 0.01$). On the other hand and in sharp contrast with current understandings, hierarchical analyses revealed no significant inter-location genetic heterogeneity and hence no IBD. Instead, genetic variation among temporal samples within sites clearly exceeded the geographical component. Our results provide support for the panmixia hypothesis and emphasize the importance of temporal replication when assessing population structure of marine fish species.

Keywords: *Anguilla anguilla*; conservation; genetic structure; microsatellites; temporal variation

This chapter is in press in: Proceedings of the Biological Society of London Series B.

[‡]Contributed equally

INTRODUCTION

European eel recruitment is at a minimum, with less than 1% compared to the 1970s. Therefore, the biological status of this species has been set outside safe limits and precautionary actions must be taken immediately (Dekker, 2003). Crucial knowledge about the biology and the life cycle of eel is lacking, but genetic markers should help in assessing the partitioning of genetic stocks. Such information is necessary to be able to develop a global management plan for this dangerously declining species.

According to the panmixia hypothesis, all European eels (*Anguilla anguilla* L.; Anguillidae; Teleostei) migrate to the Sargasso Sea for reproduction and constitute a single, randomly mating population. This hypothesis is supported by early genetic studies using allozyme and mitochondrial DNA markers (DeLigny & Pantelouris, 1973; Comparini *et al.*, 1977; Comparini & Rodinò, 1980; Yahyaoui *et al.*, 1983; Lintas *et al.*, 1998), which found no evidence for a spatial genetic structure. Similar results were obtained for the American eel (*A. rostrata*) (Avise *et al.*, 1986) and the Japanese eel (*A. japonica*) (Sang *et al.*, 1994), with the exception of clinal allozyme variation putatively imposed by selection (Williams *et al.*, 1973; Koehn & Williams, 1978; Chan *et al.*, 1997). Therefore, panmixia in European eel was widely accepted until three independent genetic studies recently reported evidence for a weak but significant population structure (Daemen *et al.*, 2001; Wirth & Bernatchez, 2001; Maes & Volckaert, 2002), with two of them finding evidence for isolation-by-distance (IBD) (Wirth & Bernatchez, 2001; Maes & Volckaert, 2002). The development and maintenance of such a structure requires temporal and/or spatial separation in the Sargasso Sea of spawning adult eels originating from different locations in Europe. This has to be followed by a non-random return of larvae to their parents' freshwater habitat through active swimming, seasonal changes in hydrodynamics or different pathways of the Gulf Stream (Wirth & Bernatchez, 2001; Maes & Volckaert, 2002).

A limitation of previous genetic studies on eels is the lack of temporal replication. For species with overlapping generations, such as the European eel, random allele frequency shifts among cohorts (year-classes) and sampling years are expected (Jorde & Ryman, 1995). If not accounted for, temporal genetic heterogeneity may incorrectly be interpreted as true population differentiation (Waples, 1998), particularly in situations of a suspected weak differentiation (Palm *et al.*, 2003a). Because marine fishes are believed to have huge population sizes, temporal genetic heterogeneity has frequently been overlooked as a potential confounding factor when assessing population structure. This view may not apply universally

because their genetically effective size (N_e), an important parameter determining temporal shifts in allele frequency (Jorde & Ryman, 1995), may be much lower than the census size (Hedgecock, 1994; Hauser *et al.*, 2002). To correct for this source of bias in the European eel, there is a need for combining a more extended geographical coverage, additional sampling points on the European coast and the assessment of the temporal stability in genetic structure.

Here we report results from the most extensive genetic study, with respect to the number of locations and individuals analysed, of the European eel to date. We explore whether temporal genetic variation plays a significant role in explaining the structure reported recently and whether any stable geographical component remains after correction for this source of bias. We do this on the basis of hierarchical *F*-statistics accounting for temporal genetic variation and tests for IBD. If confirmed, assessment of the true cause of genetic differentiation is needed to develop sustainable management options for this threatened species.

MATERIAL AND METHODS

Sampling procedures - Eel larvae start to metamorphose into "glass eels" as soon as they reach the continental shelf. When the glass eels migrate into coastal and inland waters to enter the main growth stage of the life cycle, they acquire pigments and are known as "yellow eels". The yellow eels then undergo a final metamorphosis into "silver eels" before they start their migration back to the Sargasso Sea for reproduction. Samples of glass, yellow and silver eels were collected from rivers along the European and African coasts between, 1994 and 2002. In total 2626 eels (62 samples) were collected at 41 locations, and we obtained temporal replicates at 12 of these sites (figure 1). In addition, 77 American eels were sampled at two locations and used as outgroups in a phenogram (see below). Detailed information about samples (year of sampling, life stage and sample size) is listed in Table 1. Glass eels sampled in a given year were considered to belong to a single cohort (Arai *et al.*, 2000). In contrast, samples of yellow and silver eels consisted of multiple cohorts. We were unable to classify these individual eels by cohort because otoliths were not collected for age determination. The inclusion of samples consisting of multiple cohorts may result in an underestimation of the temporal genetic component (Palm *et al.*, 2003b).

Genotyping procedures - We analysed the following six nuclear microsatellite loci, which all have been used to study population structure in the European eel previously: AAN 01, AAN 03, AAN 05 (Daemen *et al.*, 1997, 2001; GenBank, accession numbers U67163, U67165 and

AY028638, respectively), ARO 054, ARO 095 and ANG 151 (Wirth & Bernatchez, 2001; GenBank, accession numbers AF237896, AF237897 and AF237902, respectively).

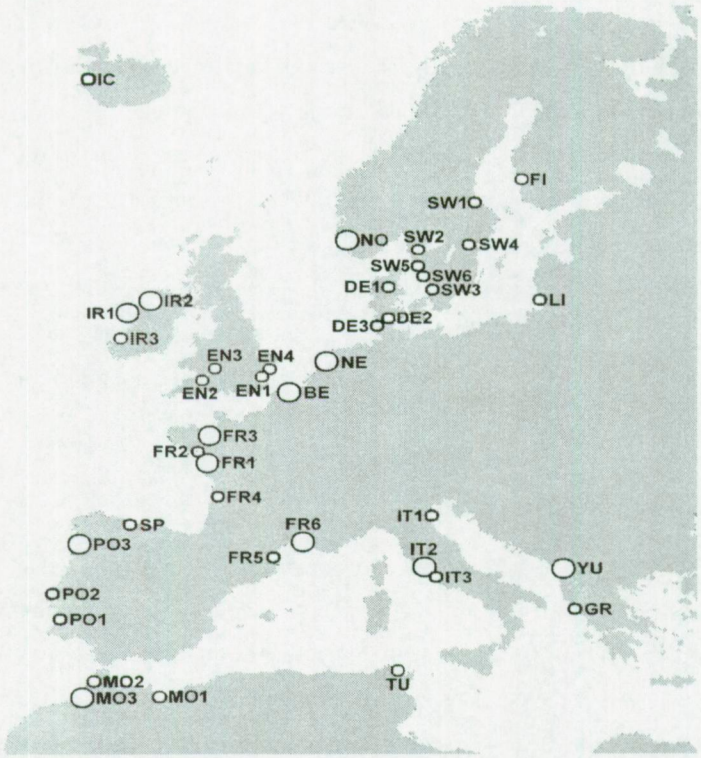


Figure 1. Sampling locations of European eel. Large dots represent localities from which temporal samples were obtained. Details for each sampling location are listed in table 1.

DNA was extracted from frozen or ethanol preserved fin or muscle tissue using a chelex protocol (Walsh *et al.*, 1991). Microsatellite analyses were conducted using multiplex polymerase chain reactions (PCR) at two laboratories following slightly different methods. At the Institute of Freshwater Research (Sweden), multiplex 1 included ANG 151, ARO 054 and ARO 095, and multiplex 2 included AAN 01, AAN 03 and AAN 05. The co-amplifications of loci were made in 25 µl volumes using Pharmacia Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech Inc, NJ, USA) and approximately 100 ng of template DNA. Primers were end labelled with fluorescent dyes.

Table 1. Sampling list (in alphabetical order) of American eel (the first two samples) and European eel including sample code (temporal samples indicated by small letters), country, locality, sampling year, life stage (G=glass eel, Y=yellow eel, S=silver eel) and sample size.

Code	Country	Sampling location	Sampling year	Life stage	Sample size
AR1	Canada	Musquash	1995	G	47
AR2	USA	St. John's River	1999	S	30
BE a,b	Belgium	IJzer	1994-2001	G-G	48-54
DE1	Denmark	Guden å	2001	G	24
DE2	Denmark	Kolding å	2001	G	24
DE3	Denmark	Vester Vedsted	2001	G	24
EN1	England	Chelmer	2002	G	24
EN2	England	Parret	1994	G	48
EN3	England	Severn	2002	G	24
EN4	England	Stour	2002	G	24
FI	Finland	Kokemäenjoki	2001	Y	45
FR1 a,b	France	Loire	2001-2001	G-S	60-50
FR2	France	Arzal	1994	G	96
FR3 a,b	France	Frémur	2000-2000	G-S	24-24
FR4	France	Gironde	2002	G	46
FR5	France	Salses Leucate	2002	G	22
FR6 a,b,c	France	Tour-du-Valat	1999-2001-2001	S-G-S	45-60-51
GR	Greece	Sagiada	2001	G	48
IC	Iceland	Ölvusá	1999	S	60
IR1 a,b,c	Ireland	Burrishoole	1999-2001-2001	S-S-G	60-60-60
IR2 a,b,c	Ireland	Erne	2001-2001-2001	G-Y-S	48-24-24
IR3	Ireland	Feale	1994	G	20
IT1	Italy	Po	1999	S	28
IT2 a,b	Italy	Martha	2001-2002	G-G	48-45
IT3	Italy	Tibern	2002	G	48
LI	Lithuania	Curonian lagoon	2001	Y	48
MO1	Morocco	Moulouya	2001	G	38
MO2	Morocco	Oued Lockkos	1994	G	48
MO3 a,b,c	Morocco	Sebou	1999-2001-2001	G-S-G	60-60-60
NE a,b,c,d, e,f,g	The Netherlands	Den Oever	1994-1999-2000-2000-2000-2001-2001	G-S-G-Y-S-G-S	48-60-48-24-22-60-60
NO a,b	Norway	Imsa	2000-2000	G-S	24-24
PO1	Portugal	Minho	2001	G	60
PO2	Portugal	Mira	1995	G	24
PO3 a,b	Portugal	Sisandro	1994-1995	G-G	24-24
SP	Spain	Asturias	1994	G	48
SW1	Sweden	Dalälven	2001	Y	24
SW2	Sweden	Ellenösjön	1998	Y	23
SW3	Sweden	Lagan	2001	G	24
SW4	Sweden	Motala	2001	Y	41
SW5	Sweden	Ringhals	2001	G	47
SW6	Sweden	Viskan	2000	G	48
TU	Tunisia	Médierda	2002	G	69
YU a,b	Yugoslavia	Bojana	2000-2001	G-G	48-48

The multiplex PCR amplifications were initiated with a denaturation step at 94 °C for 5 min followed by 27 cycles of 30 s at 94 °C, 30 s at an annealing temperature of 57 °C, and 1 min at 72 °C, and ended with a 8 min elongation step at 72 °C. Electrophoresis and size

determination of alleles was made on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) used according to the manufacturer's recommendations.

At the Laboratory of Aquatic Ecology (Belgium), multiplex 1 included ARO 095, ARO 054, AAN 05 and ANG 151, and multiplex 2 included AAN 03 and AAN 01. The co-amplifications of loci were made in 25 µl volumes including 1 × PCR buffer, 10-100 ng of template DNA, 1.5 mM $MgCl_2$, 80 µM of dNTP, 0.125 - 0.80 µM of fluorescent labeled forward and non-labeled reverse primer, and 0.5 U of Goldstar *Taq* polymerase (Eurogentec, Seraing, Belgium). The multiplex PCR amplifications were initiated with a denaturation step at 95 °C for 5 min followed by 25 cycles of 30 s at 95 °C, 30 s at an annealing temperature of 57 °C and 30 s at 72 °C, and ended with a 8 min elongation step at 72 °C. Electrophoresis and size determination of alleles was made on a LICOR automatic sequencer (Model 4200, Westburg, Leusden, The Netherlands) using a 6% acrylamide 7 M urea sequencing gel. A molecular ladder (supplied by the manufacturer) was run along with the PCR products, and allele lengths and genotypes were assessed with the GENE-IMAGIR 4.03 software (Scanalytics Inc, Fairfax, USA). A large number of randomly sampled individuals were analysed at both laboratories to calibrate the methods used for electrophoresis and size determinations of alleles.

Statistical procedures - Diversity statistics were calculated using the software GENETIX v. 4.02 (Belkhir *et al.*, 2000). Deviations from expected Hardy-Weinberg equilibrium (HWE) were calculated using GENEPOP v. 3.1d (Raymond & Rousset, 1995). The presence of null alleles was tested with the software MICRO-CHECKER v. 2.2.0 (van Oosterhout *et al.*, 2003). Population structure was studied using non-hierarchical and hierarchical *F*-statistics (Weir & Cockerham, 1984) calculated using GENETIX and ARLEQUIN v. 2.001 (Schneider *et al.*, 2000), respectively. The partitioning of genetic variance into a spatial and a temporal component was performed using the hierarchical "locus by locus AMOVA" as implemented in ARLEQUIN in combination with the "individual level" option to include also the genotypic information. Significances of *F*-statistics were evaluated through 10,000 permutations.

Tests for IBD were performed using $F_{ST}/(1-F_{ST})$ as a genetic distance (Rousset, 1997). The significances of correlations between genetic distance and nearest sea distance or difference in nearest distance to the Sargasso Sea, which is a geographical distance roughly mimicking the recruitment route of European eel, among pairwise comparisons of sampled

locations were evaluated using Mantel tests (Mantel, 1967) as implemented in GENETIX. A phenogram based on an unbiased genetic distance (Nei, 1978) was constructed using a neighbour-joining procedure in PHYLIP v. 3.6 (Felsenstein, 1993). Support for the nodes was evaluated using a bootstrap procedure with 1000 randomizations. We used two samples of American eel (table 1) as outgroups.

RESULTS

Genetic variability and Hardy-Weinberg equilibrium - All six microsatellite loci analysed were highly polymorphic, with a mean number of alleles per locus per sample (± S.D.) ranging from 7.83 (± 3.06) to 12.67 (± 5.57). Observed and expected mean heterozygosities per sample ranged from 0.53 (± 0.04) and 0.66 (± 0.13) to 0.76 (± 0.04) and 0.74 (± 0.10), respectively. Among 372 tests (62 samples × 6 loci) for HWE, 12 (3.2%) showed significant deviations from expected genotype frequencies after sequential Bonferroni (Sokal & Rolf, 1995) correction ($\alpha = 0.05$, $k = 62$), all represented heterozygote deficiencies at the loci ARO 054 and ARO 095. The risk of encountering heterozygote deficiencies as a result of large-allele dropouts has been mentioned to increase when multiplexing primers, especially for highly variable loci (O'Connell & Wright, 1997). To test this possibility, we re-amplified the two deviating loci separately for two locations, but found identical genotypes, ruling out this reason for deviation.

Samples of yellow and silver eels consisted of multiple cohorts, and allele frequency differences among these cohorts may have generated a slight heterozygote deficiency (temporal Wahlund effect, cf. Waples, 1990). However, only 33% (4 out of 12) of the deviations were found in silver or yellow eel samples. Another potential reason for deviations from HWE is the presence of null alleles. After testing all 62 samples with MICRO-CHECKER, loci ARO 054 and ARO 095 showed evidence for potential null alleles (assuming HWE within samples). Therefore, we did all analyses (except the neighbour-joining phenogram) with and without these loci to rule out the possibility that null alleles might have affected the results obtained (see below).

Spatio-temporal genetic structure - There was a low but highly significant global genetic differentiation among all 62 European eel samples ($F_{ST} = 0.0014$; $p < 0.01$) and among the 41 locations (temporal samples pooled within sites: $F_{LT} = 0.0010$; $p < 0.01$) (table 2). The global genetic differentiation was evident also in analyses excluding the two loci that deviated from

HWE (table 2). The between location differentiation remained after excluding samples with less than 48 individuals (table 2), indicating that the subtle heterogeneity observed was not due to random sampling errors associated with small sample sizes (Waples, 1998). Genetic heterogeneity between locations within individual cohorts could be studied for glass eels collected in the years, 1994, 2000, 2001 and 2002. We observed no significant differentiation between locations for the 1994, 2000 and 2001 cohorts, whereas a significant differentiation between locations was observed for the 2002 cohort (table 2).

Table 2. *F*-statistics (Weir & Cockerham, 1984) from non-hierarchical analyses of European eel samples collected in Europe and North Africa (detailed sample information is given in table 1). The analyses were based on six loci or a restricted data set with four loci (see text). Temporal samples were pooled within locations in analyses 2-4. Analyses 5-8 refer to comparisons between locations within given cohorts. Analyses 9-20 refer to comparisons between temporal samples within given locations.

Analysis	Comparison	No. samples	No. ind.	F-statistic 6 loci	F-statistic 4 loci
1	All samples	62	2626	0.0014***	0.0012**
2	All locations	41	2626	0.0010***	0.0006*
3	Locations (small samples excluded)	23	2100	0.0012***	0.0013***
4	Locations (temporal material)	12	1479	0.0012***	0.0012**
5	Locations (glass eels 1994)	8	380	-0.0002	-0.0007
6	Locations (glass eels 2000)	5	192	0.0001	0.0031
7	Locations (glass eels 2001)	17	787	0.0006	-0.0002
8	Locations (glass eels 2002)	8	302	0.0045***	0.0048**
9	France (Loire)	2	110	-0.0002	-0.0011
10	France (Frémur)	2	48	0.0080*	0.0187**
11	France (Tour du Valat)	3	156	0.0020	-0.0025
12	Netherlands (Den Oever)	7	322	0.0027**	0.0046**
13	Ireland (Burrishoole)	3	180	0.0017	-0.0009
14	Ireland (Erne)	3	96	-0.0003	0.0001
15	Morocco (Sebou)	3	180	0.0027*	0.0030
16	Norway (Imsa)	2	48	-0.0101	-0.0113
17	Belgium (IJser)	2	102	0.0024	0.0037
18	Italy (Martha)	2	93	-0.0053	-0.0038
19	Yugoslavia (Bojana)	2	96	-0.0011	0.0011
20	Portugal (Sisandro)	2	48	-0.0004	-0.0024

*, $p < 0.10$; **, $p < 0.05$; ***, $p < 0.01$.

There were also indications of genetic heterogeneity among temporal samples within locations (table 2), prompting for a hierarchical analysis to account for this variation when evaluating spatial genetic structure. When including only the 12 locations for which temporal samples were available, genetic differences between temporal replicates collected at

individual sites explained a larger proportion of the total genetic variance than did differences between geographical locations (table 3). In fact, the between location source of variance was not significantly different from zero. The temporal component was even stronger when excluding samples with less than 48 individuals, but only approached significance in analyses based on 4 loci (table 3).

Table 3. Tests for spatial and temporal genetic variation in the European eel using *F*-statistics from hierarchical analyses (AMOVA) based on either six or four loci (*p*-values within parentheses). The "12 locations" analyses include all locations from which temporal samples were obtained, whereas samples with less than 48 individuals were excluded in the "7 locations" analyses to check for potential sampling errors associated with small sample sizes (see text).

				F-statistic	
Data set	No. samples	No. ind.	No. loci	Among locations	Among temporal samples
12 locations	33	1479	6	0.0007 (0.11)	0.0012 (0.05)
			4	0.0007 (0.21)	0.0015 (0.11)
7 locations	19	1055	6	0.0006 (0.16)	0.0017 (0.03)
			4	0.0001 (0.44)	0.0020 (0.15)

Isolation-by-distance and cluster analysis - We found no correlation between genetic distance and nearest sea distance or difference in distance to the Sargasso Sea among pairwise comparisons between sampled locations, neither for the complete data set (all 41 locations) nor for a data set in which only glass eels were included to avoid potential biases due to secondary movements of adults and translocation activities (table 4). Also, there was no correlation between genetic and geographical distances among pairwise comparisons of glass eel samples within individual cohorts except for cohort 2000 (table 4). The distant Yugoslavian sample was the only contributor to the IBD pattern observed for cohort 2000, as this sample was involved in the few significant pairwise comparisons within this cohort (data not shown). No significant differences were observed in pairwise comparisons involving the other glass eel samples from cohort 2000. The absence of a consistent IBD pattern was supported by an AMOVA in which locations (temporal samples pooled) were grouped according to three main ocean basins (North Atlantic/Baltic Sea, Atlantic Basin, and Mediterranean Sea). These groups have been mentioned as possible genetic units responsible for the IBD pattern observed previously (Wirth & Bernatchez, 2001, 2003; Maes & Volckaert, 2002). No genetic differentiation was observed between the three groups (F_{GT} = -0.0001; p = 0.695), whereas a weak but significant heterogeneity (most likely

attributed to temporal genetic variation, see above) was observed among locations within groups ($F_{LG} = 0.0010$; $p = 0.020$).

Table 4. Tests for isolation-by-distance in the European eel. Results from correlations between genetic distance ($F_{ST}/1\text{-}F_{ST}$) and (1) nearest sea distance or (2) difference in nearest distance to the spawning area in Sargasso, for pairwise comparisons between all sampled locations (temporal samples pooled), between glass eel locations (temporal samples pooled), and between glass eel samples collected in four years. The analyses were carried out using all six loci and a restricted data set with four loci (see text).

			6 loci		4 loci	
Data set	No. samples	Analysis	Pearson's r	p	Pearson's r	p
Complete data	41	1	-0.09	0.84	-0.02	0.64
		2	-0.09	0.88	-0.05	0.75
Only glass eels	34	1	0.04	0.32	0.06	0.22
		2	0.01	0.45	0.03	0.32
Cohort 1994	8	1	0.03	0.43	-0.05	0.57
		2	-0.31	0.88	-0.13	0.70
Cohort 2000	5	1	0.58	0.05	0.53	0.05
		2	0.12	0.37	0.09	0.51
Cohort 2001	17	1	-0.13	0.83	-0.11	0.83
		2	-0.01	0.53	0.04	0.35
Cohort 2002	8	1	0.04	0.42	-0.01	0.48
		2	-0.02	0.50	-0.01	0.52

Further, a neighbour-joining phenogram based on Nei's (1978) unbiased genetic distance between all samples did not show any clustering of temporal samples within locations, or locations within regions (figure 2). The European eel separated from the two American eel samples in all bootstrap replicates, but no nodes within the European eel were supported in more than 57% of the bootstrap replicates.

DISCUSSION

The results presented here clearly show that temporal replication is crucial in the study of genetic differentiation in marine organisms. On the basis of hierarchical F-statistics accounting for temporal genetic variation and tests for IBD, we conclude that European eels sampled along the coasts of Europe and Africa most likely belong to a single, spatially homogeneous population. Hence, our study re-opens the debate about panmixia in this species and emphasizes the need for a deeper look into European eel using a standardised sampling approach and multiple markers.

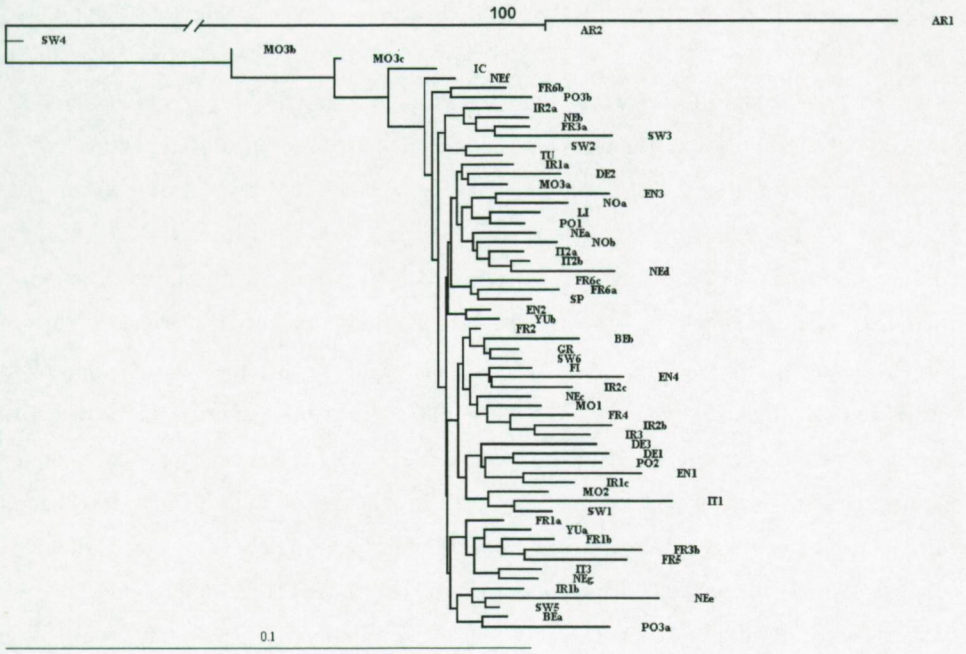


Figure 2. Neighbour-joining phenogram based on Nei's (1978) unbiased genetic distance among two samples of American eel and 62 samples of European eel. The designations of samples refer to those listed in table 1 (temporal samples within locations are identified by small letters). Note the broken branch separating the two species.

Sources of genetic variation

We found a significant global genetic differentiation between all European eel samples (F_{ST} = 0.0014), which is close to the values previously reported using the same type of markers (Daemen *et al.*, 2001; Wirth & Bernatchez, 2001). When pooling temporal samples within locations, the overall differentiation decreased, albeit remaining significant (F_{LT} = 0.0010). This is an indication that temporal genetic variation might explain a significant amount of the total genetic variance. In addition, splitting the samples into cohorts lowered the differentiation value and increased the *p*-value above significancy for most groups analysed, emphasizing again the temporal nature of the global differentiation observed among samples. In the hierarchical analysis of molecular variance, the proportion of the total genetic variance explained by differences between temporal samples within locations was at least twice as high as the proportion due to spatial differences. For individual locations, the strongest indications of temporal genetic variation were found in those cases where temporal samples were relatively large and were separated in time by many years or consisted of eels of different life

stages (large differences in age), which is in close agreement with previous theoretical and empirical work (Jorde & Ryman, 1995; Palm *et al.*, 2003b). However, because of small sample sizes, the statistical power in the within location analyses becomes low, which might explain the many non-significant comparisons despite relatively high differentiation values.

It is important to note that our findings do not preclude the presence of a spatial genetic structure in the European eel. However, the results suggest that the global genetic differentiation that may possibly exist must be extremely weak. The significant differentiation between glass eel samples in 2002 may point to a spatial structure. However, the surface of the Sargasso Sea is huge (5.2×10^6 km^2) and due to its heterogeneous hydrographical structure (McGillicuddy *et al.*, 2001; Knights, 2003), the mating patterns and the dispersal of larvae to the coasts of Europe may not be completely random. Thus, the differences observed among glass eel samples from 2002 may well be the consequence of that eels caught at different sites in Europe were derived from different (finite) sets of parents (Allendorf & Phelps, 1981; Waples, 1998). This might also explain the IBD signal for cohort 2000, which was mainly a result of that the distant Yugoslavian sample differed slightly from the other samples belonging to this cohort. Nevertheless, the lack of a global differentiation among glass eels collected in 1994, 2000 and 2001, in combination with the non-significant hierarchical *F*-statistic between geographical locations and the absence of an IBD pattern in the total material and in three out of four cohorts strongly indicate that the occasional differences observed are not consistent over time.

Comparison with previous studies

Temporal delay in the arrival of adults from different latitudes on the East-Atlantic continental shelf to the breeding site in the Sargasso Sea, possibly in conjunction with spatial separation of spawning sites, has been proposed as an explanation for the genetic structure observed previously (Wirth & Bernatchez, 2001; Maes & Volckaert, 2002). If true, this temporal allopatry in combination with non-random return of larvae may generate an IBD pattern among eels caught in Europe, with some degree of genetic exchange between neighbouring populations due to some overlap in spawning time, but restricted gene flow between distant populations. In sharp contrast to previous studies, we found no evidence for IBD. When we applied a geographical clustering into three groups as suggested in earlier studies (Wirth & Bernatchez, 2001, 2003; Maes & Volckaert, 2002), no significant differentiation could be found between the groups (North Atlantic/Baltic Sea, Atlantic Basin,

and Mediterranean Sea). In addition, we could not detect any geographical grouping of European eel samples in a neighbour-joining phenogram. Wirth and Bernatchez (2001, 2003) presented similar analyses in which Mediterranean samples and samples from the North Sea and the Baltic Sea were proposed to form distinct clades, but these conclusions were based on very low bootstrap values. In the present study, no nodes within the phenogram were supported in more than 57% of the bootstrap replicates, and no obvious structure was evident even if boostrap values were not taken into account.

The contrasting results between this and previous studies regarding the IBD pattern is puzzling. One reason could be differences between studies in statistical power. However, we find this explanation quite unlikely, as the number of samples is higher in the present study, and the sample sizes in many cases exceed those in previous studies. The splitting of samples into cohorts will of course decrease statistical power in subsequent analyses of IBD. Also, the use of only four loci in the restricted data set likely reduces power to detect IBD. However, the absence of even a slight signal of IBD (see Pearson's correlations in table 4) in three out of four cohorts indicates that differences in statistical power is a less plausible explanation for the contrasting results. Also, if it exists, IBD would have been discovered in the analyses including all 41 locations and all glass eel locations.

Instead, we argue that differences in sampling procedures might explain the discrepant results of this and previous studies. We note that samples included in the study by Wirth and Bernatchez (2001) were collected in the same year but consisted of eels of different ages. The five northernmost samples included older yellow and silver eels whereas the eight southern samples consisted of newly recruited glass eels. In the presence of even a slight temporal genetic heterogeneity, such a sampling scheme may produce a spurious correlation between genetic and geographical distance. However, an additional analysis in which the northernmost samples were excluded also indicated presence of IBD (T. Wirth, personal communication) although the correlation was weaker and only approached significance. Inclusion of one or a few distant samples that deviate from the others because they derived from different (finite) sets of parents (see above) could also result in a spurious correlation between genetic and geographical distance. Similar artefacts may explain the observations of Maes and Volckaert (2002), as their study also relied on samples collected in different years and only one distant sample (consisting of eels that differed in age from all other sampled eels) was the main contributor to the observed IBD pattern.

One caveat applies to our conclusion of no genetic substructuring within the European eel, namely that no or a very weak geographical differentiation at neutral loci does not preclude

the existence of more pronounced differences at loci affected by selection (Cousyn *et al.*, 2001; Koskinen *et al.*, 2002). Because selected and non-selected genes can have different effective migration rates, adaptive differences could in theory persist in spite of significant neutral gene flow. Thus, there is always a risk that neutral markers like microsatellites may fail to document population differences.

Effective population size and temporal genetic variation

Although we cannot properly estimate N_e from our temporal genetic data without basic demographic information (Jorde & Ryman, 1995), the observed temporal genetic heterogeneity indicates that the effective size of the European eel stock might not be as large as previously thought. Wirth and Bernatchez (2003) used the procedure of Beaumont (1999), which estimates several genealogical and demographic parameters from microsatellite data using Bayesian statistics, and presented values of the current effective size of the European eel stock that ranged between 2,100 and 12,000. These values correspond to remarkably low numbers of spawning eels that succeed in reproduction each year. However, results obtained using this method should be viewed with caution as the analysis assumes knowledge about the mutation rate, which may differ considerably between loci, and that the microsatellite loci used have evolved according to a strict stepwise mutation model (Beaumont, 1999).

On the other hand, these figures may not be unrealistic as the estimated effective size of marine fishes can be several orders of magnitude less than the actual number of adults present in the population (Hauser *et al.*, 2002). The genetic "sweepstake" hypothesis (Hedgecock, 1994), which has affinities with the ecological match/mismatch hypothesis (Beaugrand *et al.*, 2003), was proposed to explain extremely low effective to census size ratios in highly fecund marine species, and states that many families do not recruit because their larvae do not end up in the right environment at the right time to survive critical life stages. Given the very distant larval migrations in an environment where currents and primary production vary seasonally and interannually (Desaunay & Guérault, 1997; McGillicuddy *et al.*, 2001), the European eel may qualify for such a scenario.

Implications

Our findings have implications for the sustainable management of the European eel, whose abundance has declined steadily since the late 1970s (Dekker, 2003). Many factors might be involved, such as long-term oceanic and climatic changes (Wirth & Bernatchez, 2003),

overfishing, pollution, diseases and the destruction and overexploitation of freshwater habitats (Knights, 2003). A global management action developed for the Eastern-Atlantic shelf (including the Mediterranean) is supported by the present results, rejecting a strictly local approach. Future genetic studies on eel should focus on the collection of data necessary for a proper estimation of N_e. Also, genetic monitoring may facilitate the detection of changes in population genetic dynamics, such as an increase in the magnitude of temporal allele frequency shifts resulting from a reduction in N_e. Our results also have implications for marine fishes in general. To avoid extinction of local populations, the successful management of commercial marine fish species requires sufficiently large spawning stocks and a thorough understanding of the dynamics of their population genetic structure (Nielsen, 2001; Myers & Worm, 2003). The detection of temporal genetic variation in a presumably panmictic species emphasizes the need to control for this source of variation when evaluating subtle population structure of threatened marine fishes.

ACKNOWLEDGMENTS

We thank the following people for their help in obtaining samples: B. Jonsson, R. Poole (Marine Institute, Newport, Ireland), W. Dekker, J. Van Willigen, C. Belpaire, P. Elie, A. Correia, A. Crivelli, A. Yahyaoui, J. Aoyama, D. Evans, M. I. Pedersen, E. Ciccotti, B. Jessop, B. Ragnarsson, J. Tielman, K. Torildsson, B. Fagerholm, J. Andersson, E. Feunteun, R. Lecomte-Finiger, J. Backer, A. Vøllestad, R. Rosell, R. Andjus, A. Hegedis, S. Zobola, G. Minos, J. Tulonen, L. Lozys, E. Hahlbeck, I. Rahhou, W.-N. Tzeng, B. Knights, P. Wood, R. Wright, S. Magdy, and J. Reynolds (European Glass Eel Bank at Trinity College, Dublin). M. Gehlin and T. Soler prepared the map and measured geographical distances. We also thank J. Höglund, M. Hansen, R. Waples, S. Palm and T. Wirth for constructive criticism on earlier drafts. The work of J.D., L.J., H.W. and T.J. was financed by Formas, the National Board of Fisheries in Sweden, and the Financial Instrument for Fisheries Guidance (FIFG). G.E.M. was supported by an IWT (Institute for the Promotion of Innovation by Science and Technology in Flanders) graduate scholarship and EU contract EELREP (Q5RS-2001-01836).

CHAPTER V

Evidence for Isolation-by-Time in the European eel

Maes G. E., Pujolar J.M., Hellemans B. and Volckaert F.A.M.

SUMMARY

Life history traits of highly vagile marine species, such as adult reproductive success and larval dispersal, are usually determined by oceanographic and climatic forces. Nevertheless, marine organisms may show restricted dispersal in time and space. Patterns of Isolation-by-Distance (IBD) are commonly observed in marine species. If spawning time is a function of geographical location, temporal and spatial isolation, however, can easily be confounded or misinterpreted. In this study, we aimed at discriminating between various forces shaping the genetic composition of recruiting juveniles of the European eel (*Anguilla anguilla* L.). By controlling for geographical variation, we assessed temporal variation and tested for Isolation-by-Time (IBT) between spawning cohorts within and between years. Using morphometric traits, as well as 12 polymorphic allozyme and eight variable microsatellite loci, we show that genetic differentiation was low (F_{ST} = 0.2 % - 0.9%) and significant between cohorts. Regression between genetic and temporal distance, however, was consistent with an inter-annual pattern of Isolation-by-Time. Our data suggest that the population dynamics of the European eel are governed by a double pattern of temporal variance in genetic composition: (1) a broad scale Isolation-by-Time of spawning cohorts, possibly as a consequence of the large migration loop in anguillids or strong variance in annual adult reproductive success; and (2) a small scale variance in reproductive success (genetic patchiness) among monthly spawning cohorts, most likely originating from seasonally oceanic and climatic forces. The consistency of the IBT remains to be verified in spawning or migrating aged adults to confirm its stable or transient nature.

Keywords: clinal variation; genetic patchiness; Isolation-by-Distance; member-vagrant; North Atlantic Oscillation; population genetics; relatedness; temporal genetic variation

This chapter is presented as an unpublished manuscript.

INTRODUCTION

Widely distributed species are rarely fully panmictic, but are often divided into subgroups in a pattern that can be described by one of the population models, such as the island model, stepping stone model or the isolation by distance model. The genetic architecture of natural populations is the outcome of factors such as population size, individual dispersal, behaviour, assortative mating, reproductive success and survival (Rousset, 1997; Avise, 2004). In order for populations to diverge, strong temporally stable barriers to gene flow are required to prevent the dispersal of migrants. If separation is incomplete, some level of gene flow may occur between spatially adjacent populations, creating a pattern of Isolation-by-Distance (IBD) (Rousset, 1997). An additional population structure may occur in taxa, of which populations are composed of individuals or groups reproducing at different times of the year or season (Hendry & Day, 2005). Many examples exist of species exhibiting multiple spawning periods within a year, possibly creating a succession of temporally instead of spatially separated populations. Gene flow between such temporally separated populations may create a pattern of Isolation-by-Time (IBT) (Hendry & Day, 2005). Although individual reproductive output may vary, time at reproduction is thought to be highly heritable, being influenced by genetic (breeding value) as well as environmental factors. IBT can be tested by indirect genetic measures of dispersal in time, such as assignment of individuals to populations separated in time or a correlation analysis between temporal and genetic distance (Rousset, 1997; Hansen *et al.*, 2001; Berry *et al.*, 2004). To date, no IBT analysis has been performed comparing early and late spawners in fish (Brykov, 1999; Fillatre, 2003, Hendry *et al.*, 2004).

Marine organisms were not included in a review on IBT by Hendry & Day (2005). Many species show only subtle spatio-temporal variation and exhibit often ill-defined or variable spawning regions (Waples, 1998). Their life history traits, such as adult reproductive success, larval dispersal potential, post-hatching survival and early life stage selection, are most often driven by oceanographic and climatic forces (Palumbi, 1994; Waples, 1998; Cowen *et al.*, 2000; Largier, 2003). The unpredictability of the marine environment may lead to a differential contribution of parents and subsequent temporal variation in the genetic composition of the recruits (Hedgecock, 1994; David *et al.*, 1997; Li & Hedgecock, 1998; Johnson & Wernham, 1999; Planes & Lenfant, 2002). Under the hypothesis of "sweepstakes reproductive success" (Hedgecock 1994), chance events determine which adults are successful in each spawning event. Hedgecock (1994) attributed the variation in reproductive

success of adults to spatio-temporal variation in oceanographic conditions, occurring both within and among seasons. Fluctuations in recruitment success and population persistence are highly dependent on a match with food at the spawning place (match-mismatch hypothesis; Cushing, 1990, 1996), the degree of stability of retention zones (member-vagrant hypothesis; Sinclair, 1988) and social transmission of migration patterns and spawning areas from adults to recruiting individuals (adopted-migrant hypothesis, McQuinn, 1997). Marine species may be forced to split their reproductive effort among several spawning events within a reproductive season, a process comparable to bet-hedging (Flowers *et al.*, 2002). Nevertheless, they may also show a restricted dispersal in time and space. Marine organisms actively choose spawning sites with a high ecological value to maximize the survival of larvae. Larval retention zones will subsequently provide hatchlings of adequate food supplies for weeks to months until they reach nurseries habitats (Sinclair, 1988). These larval retention zones can be spatially delineated or one zone can be used by temporally separated spawning cohorts (Ruzzante *et al.*, 1996; Stepien *et al.*, 1999; Hoarau *et al.*, 2002). The degree of spatio-temporal overlap between spawning groups will determine the level of geographical or inter-cohort genetic differentiation between populations. Although Isolation-by-Distance has been routinely observed between marine populations (Pogson *et al.*, 2001; Wirth & Bernatchez, 2001; Maes & Volckaert, 2002; O'Reilly *et al.*, 2004), temporal and spatial isolation can easily be confounded or misinterpreted, if spawning time is a function of geographical location. If some distinct populations overlap strongly in spawning time and subsequently mix, a temporal Wahlund effect can be observed within samples (Wahlund, 1928; Hoarau *et al.*, 2002). If temporal overlap is small, a stable pattern of Isolation-by-Time (IBT) may be generated between consecutive spawning cohorts (Hendry & Day, 2005). The stability of this pattern depends on the heritability of reproductive time in early and late spawners at a spawning site. Adults with a given reproductive value will generate offspring of an intermediate reproductive value. The lower the heritability of spawning time, the higher the temporal gene flow through dispersal (Hendry & Day, 2005). So far, Isolation- by-Time has not been specifically tested in marine organisms, although several studies successfully investigated temporally separated populations of cod, red drum, plaice and herring, where successive larval or adults samples were collected in possible retention zones (Ruzzante *et al.*, 1996, 1999; Beacham *et al.*, 2002; Hoarau *et al.*, 2002; McPherson *et al.*, 2003).

The genetic consequences of a high variance in reproductive success is the induction of an unpatterned genetic constitution of recruits (genetic patchiness), that counteracts a population structure following an IBT pattern, especially if patchiness surpasses forces restricting

dispersal in time. Due to strong intra- and inter-locus variance and random sampling errors when analysing marine species (Waples, 1998), sampling temporally replicated larval cohorts over a broad geographical region and integrating hydrodynamic oceanic forces into genetic structure analyses may be needed to detect a pattern of IBT despite genetic patchiness.

The life-history of the catadromous European eel (*Anguilla anguilla* L.) strongly depends on oceanic conditions; maturation, migration, spawning, larval transport and recruitment dynamics are completed in the open ocean (Tesch, 2003). Its life cycle (also called "migration-loop") follows faithfully the North-Atlantic gyral system (Tsukamoto *et al.*, 2002). Partially mature adults leave the continental rivers at different times, strongly dependent on lunar phase and atmospheric conditions (Desaunay & Guérault, 1997; Okamura *et al.*, 2000; Tesch, 2003), swim southward using the Canary and North-Equatorial currents and arrive six to seven months later at the Sargasso Sea to spawn and then die. The leptocephali larvae are transported along the Gulf Stream and North-Atlantic Drift for a journey of eight to nine months back to the eastern Atlantic coast (Lecomte-Finiger, 1994; Arai *et al.*, 2000), where they metamorphose to glass eels, ascent rivers and grow till partial maturity, six to ten years later (Tesch, 2003). Despite the key biological importance of the marine phase (Knights, 2003), researchers have dedicated more efforts to understand the freshwater phase of its life-history. A better understanding of the biology of the European eel becomes increasingly urgent, as the current recruitment level has dropped to only 1% of the 1960 recruitment level, bringing the species to the brink of extinction (Dekker, 2003).

The commonly accepted view of a panmictic population structure of European eel, based on oceanographic (Sinclair, 1988; Tesch, 2003) and genetic features has been recently challenged in three independent studies (Daemen *et al.*, 2001; Wirth & Bernatchez, 2001; Maes & Volckaert, 2002). Wirth & Bernatchez (2001) and Maes & Volckaert (2002) detected a pattern of Isolation-by-distance (IBD) and suggested a spatio-temporal separation of spawning populations with some degree of gene flow. This pattern proved, however, to be unstable over time (Dannewitz *et al.*, 2005). As the life history of European eel exhibits the largest migration-loop of any anguillid, there is a possibility that individuals from geographically separated regions differ in arrival time at the spawning site (Tsukamoto *et al.*, 2002). Genetic analysis of consecutive spawning or larval cohorts at several geographical sites allow for testing whether such IBD pattern originates from a combination of spatial and temporal differentiation.

In the present study, we used a standardized sampling strategy to monitor recruiting glass eels at six geographical locations over three consecutive years in the Atlantic Ocean and the

Mediterranean Sea. We first aimed at detecting, partitioning and quantifying the spatial and temporal component of genetic variation in glass eels. We subsequently tested for temporal genetic variation among glass eel cohorts following an IBT pattern within and between years, while controlling for spatial differentiation. Finally, we assessed whether oceanic conditions may play a role in the average relatedness within and between cohorts, by testing the genetic patchiness hypothesis. If the signal of an IBT pattern and genetic patchiness are comparable, no stable heritable temporal population structure can be maintained. On the other hand, if an IBT signal exceeds genetic patchiness, temporal separation of spawning cohorts may be maintained through time and possibly lead to diachronically self-sustaining spawning populations within the European eel.

MATERIAL AND METHODS

Material - We collected a total of 1012 glass eels over a period of three years at six different geographical sites. In total 17 samples were taken between 1999 and 2003 at 5 Atlantic (Iceland, Ireland, The Netherlands, Western-France and Morocco) and one Mediterranean (Southern France) sites. Glass eels were sampled during the main glass eel arrival peak (approx. 2-3 months) to increase comparability of cohorts between years (Table 1). Each cohort was characterized by standard length (L) and weight (W) measurements (see Table 1 for details).

Allozyme genotype detection - All individuals were analysed for allozyme variation using Cellulose Acetate Gel Electrophoresis (CAGE, Harris & Hopkinson, 1976; Richardson *et al*, 1986). Tissue extraction, electrophoresis, procedures for visualizing proteins and buffer systems used (Tris Glycine (TG) and Tris Malate (TM)) are described in Maes & Volckaert (2002). Eight enzymatic systems, coding for 12 loci, were examined: aspartate aminotransferase (*AAT-1**, *AAT-2**, *AAT-3**, EC 2.6.1.1, TM), alcohol dehydrogenase (*ADH**, EC 1.1.1.1, TG), glucose-6-phosphate isomerase (*GPI-1**, *GPI-2**, EC 5.3.1.9, TG), isocitrate dehydrogenase (*IDHP**, EC 1.1.1.42, TM), malate dehydrogenase (*MDH-2**, EC 1.1.1.37, TM), mannose-6-phosphate isomerase (*MPI**, EC 5.3.1.8, TG), 6-phosphogluconic dehydrogenase (*PGDH**, EC 1.1.1.44) and phosphoglucomutase (*PGM**, EC 5.4.2.2, TG). Genetic nomenclature followed the suggestions of Shaklee *et al.* (1990). Allele assignment was carried out comparing the relative distance with the most common allele (**100*).

Table 1: Summary of glass eel (*Anguilla anguilla*) samples including country, sampling site, geographical coordinates, sampling period, number of individuals analyzed (N), mean length in cm (L), weight in g (W) and condition (K). Standard deviations are given in parentheses.

Country and Sampling site	Latitude	Longitude	Sampling period	Code	N	L	W	K
Iceland (IC)	64° 23'N	21° 22'W	01/07/2001	IC01g	60	6.92 (0.32)	0.30 (0.06)	0.91 (0.16)
Vogslækur			01/07/2002	IC02g	60	6.86 (0.31)	0.25 (0.04)	0.76 (0.10)
			01/06/2003	IC03g	52	6.80 (0.37)	0.24 (0.08)	0.76 (0.17)
Ireland (IR)	53° 55'N	09° 55'W	01/06/2001	IR01g	60	6.46 (0.28)	0.33 (0.05)	1.23 (0.14)
Burrishoole			01/03/2002	IR02g	60	6.84 (0.40)	0.35 (0.06)	1.07 (0.13)
			01/04/2003	IR03g	60	7.04 (0.32)	0.34 (0.05)	0.97 (0.13)
The Netherlands (NL)	53° 01'N	05° 13'E	01/06/2001	NL01g	60	6.36 (0.39)	0.24 (0.05)	0.92 (0.18)
Den Oever			01/05/2002	NL02g	60	7.24 (0.44)	0.33 (0.06)	0.88 (0.13)
			01/05/2003	NL03g	60	7.50 (0.30)	0.27 (0.05)	0.65 (0.08)
W France (FR)	47°12'N	01° 44'W	01/04/2001	FR01g	60	6.48 (0.37)	0.31 (0.05)	1.14 (0.22)
Loire estuary			01/01/2002	FR02g	60	7.36 (0.28)	0.47 (0.07)	1.19 (0.15)
			01/03/2003	FR03g	60	7.74 (0.33)	0.38 (0.06)	0.81 (0.08)
S France (TV)	43° 33'N	04° 38'E	01/01/2001	TV01g	60	6.21 (0.31)	0.31 (0.09)	1.29 (0.34)
Tour du Valat			01/02/2002	TV02g	60	6.73 (0.27)	0.39 (0.08)	1.27 (0.20)
			01/03/2003	TV03g	60	6.28 (0.33)	0.25 (0.05)	0.99 (0.13)
Morocco (MA)	34° 16'N	06° 34'W	01/05/1999	MA99g	60	/	/	/
Oued Sebou			01/05/2001	MA01g	60	5.99 (0.25)	0.28 (0.06)	1.29 (0.23)

DNA purification and microsatellite amplification – All allozyme genotyped individuals (excepted IC02g) were also screened for microsatellite variation. DNA purification and multiplex-loci PCR amplification are described in Dannewitz *et al.* (2005). Eight polymorphic microsatellite loci were examined: AAN 01, AAN 03, AAN 05 (Daemen *et al.*, 2001); ARO 095, ARO 054, ARO 063, ANG 151 and ANG 075 (Wirth & Bernatchez, 2001). Electrophoresis and allele size determination were performed on an automated LICOR (Model 4200, Westburg, Leusden, The Netherlands) using a 6% acrylamide 7 M urea sequencing gel. A molecular ladder (supplied by the manufacturer) was run along with the PCR products, and allele lengths and genotypes were assessed with the GeneImagIR 4.03 software (Scanalytics inc, Fairfax, USA).

Morphometric analysis - All individuals were measured for standard length (L) and body weight (W). Fulton's condition factor ($K = 1000(W/L^3)$) was calculated for each individual, where L is standard length in cm and W is body weight in g. The condition factor is based on the analysis of length-weight data, assuming that heavier individuals at a given length have a better condition. Inter-annual and spatial differences in morphometric measures were tested

using a factorial analysis of variance (ANOVA). The relation between oceanic factors and morphometry was tested using regression analyses (Pearson's correlation coefficient). These factors were the winter North Atlantic Oscillation Index (NAOI) (from http://www.cgd.ucar.edu/~jhurrell/nao.html) and the 200 m isobath distance (the edge of the continental slope is known to induce metamorphosis (Tesch, 2003)). The winter NAO index was lagged by one year, as the mean larval transport of European eel larvae takes approximately 249 ($\pm$ 23) days (Arai *et al.*, 2000). All comparisons were performed using the STATISTICA 6.1 application (Statsoft).

Data analysis of genotypes - Genetic diversity estimates such as the level of polymorphism, and observed and expected heterozygosity (H_o and H_e) were calculated in GENETIX version 4.05 (Belkhir *et al.*, 1999). Statistical comparisons of allelic richness (R) and gene diversity (H_S) between years and locations, as well as departures from Hardy-Weinberg equilibrium (F_{IS}) were calculated using the software FSTAT version 3.9.5 (Goudet, 1995). Genetic differentiation was characterized using hierarchical F-statistics (θ, Weir & Cockerham, 1984) as implemented in the GENETIX 4.05 software package (Belkhir *et al.*, 1999). Significance tests were assessed with permutation tests (1000 replicates). In all cases significance levels were corrected for multiple comparisons using a sequential Bonferroni correction (Rice, 1989). Genetic variability was partitioned into a within geographical site (F_{SC}, temporal) and between geographical site component (F_{CT}, geographical) using an Analysis of Molecular Variance (AMOVA) in ARLEQUIN version 2.0 (Schneider *et al.*, 2001). Pairwise genetic distances were calculated following Cavalli-Sforza & Edwards (1967) chord distance (D_{CE}) and a Neighbour-joining dendrogram with branch boostrap values (1000 iterations) was constructed using the software package PHYLIP 3.5 (Felsenstein, 1996). We used a non-parametric Kruskal-Wallis procedure to compare pairwise F_{ST} estimates between samples separated by 0, 1 or 2 years, implemented in STATISTICA 6.1 (Statsoft). Isolation-by-Distance (IBD) and Isolation-by-Time (IBT) were tested using single and partial Mantel tests (Mantel, 1967) implemented in GENETIX and FSTAT 3.5, by correlating coastal distance (between sites) and temporal distance (years and days since recruitment) versus $F_{ST}/1\text{-}F_{ST}$ as suggested by Rousset *et al.* (1997) and Hendry & Day (2005). Average relatedness (r_{xy}, Queller & Goodnight, 1989) was calculated within samples, between geographical samples and between temporal samples (pooling geographical samples) using the program IDENTIX (Belkhir *et al.*, 2002). Significance of the means was calculated by 1000 Markov-Chain permutations over the alleles, yielding a distribution of mean relatedness values under the

assumption of no relatedness (panmixia). ANOVA tests were used to test for statistical differences between the mean pairwise differences of each sample in STATISTICA 6.1 (Statsoft).

RESULTS

Morphometric data – Highly significant differences were detected between geographical sites and between years in the variables length and weight (Figure 1 a, b, c). In all comparisons, there was a significant effect of sampling location ($F[12, 2349.8] = 81$, $p < 0.001$) and sampling year ($F[6, 1776] = 119$, $p < 0.001$). The interaction sampling *site*year* was also highly significant ($p < 0.001$). When tested separately, inter-annual variance was the highest in Ireland, The Netherlands and Western-France ($p < 0.001$), while Iceland and Southern France showed a more constant pattern in length ($p > 0.05$).

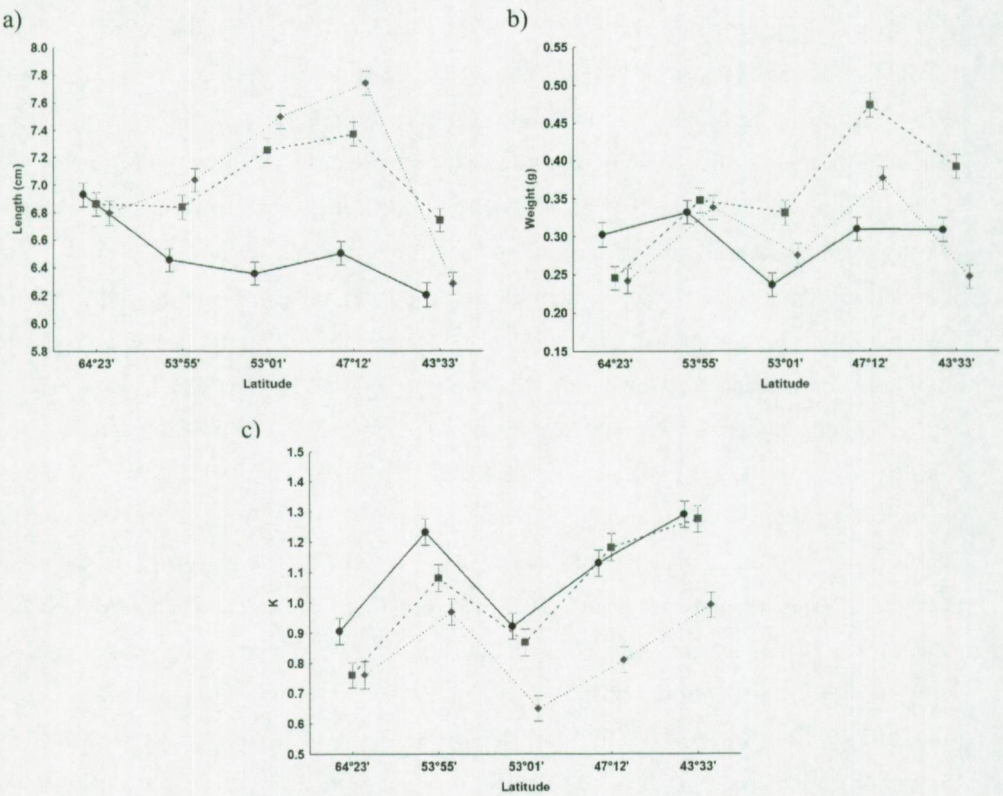


Figure 1 : a) Length (cm), b) Weight (g) and c) Fulton's condition index (K) of glass eel (*Anguilla anguilla*) collected in 2001, 2002 and 2003. Vertical bars represent 95% confidence intervals. Circles: 2001; squares: 2002; diamonds: 2003.

Overall, there was an increase in length from 2001 to 2003. The condition index (K) showed a very congruent pattern between years, with a downward trend over the years. Iceland and The Netherlands showed the lowest condition over the three years. The highest differences were observed between sites. The winter North Atlantic Oscillation Index is significantly negatively correlated to length ($r = -0.42$, $p < 0.001$) and weight ($r = -0.30$, $p < 0.001$), but positively correlated to the condition index ($r = 0.14$, $p < 0.001$). Length and condition were correlated to the distance from the 200 m-isobath line of the continental shelf ($r = 0.341$, $p < 0.001$ and $r = -0.51$, $p < 0.001$, respectively).

Genetic diversity within and between sites – A total of 12 polymorphic allozyme loci were screened, with observed and expected multilocus heterozygosities per sample ranging from 0.156 to 0.212 and from 0.165 to 0.20, respectively (Table 2). Half of the loci were moderately polymorphic, while many loci exhibited rare alleles. The number of rare alleles (calculated as the difference between the $P_{(0.95)}$ and $P_{(0.99)}$ level of polymorphism) showed a negative correlation with latitude (spearman rank correlation: $r = -0.69$, $p < 0.05$), pointing to a lower number of rare alleles at higher latitudes. Allelic richness ranged from 2.48 to 3.15 and increased in successive years (from 2001 to 2003) together with gene diversity (H_S) (data not shown). The Western French samples exhibited the lowest heterozygosity values (H_o and H_S, $p < 0.05$) and lower allelic richness compared to other sites. No other significant differences were found. No population departed significantly from Hardy-Weinberg expectations.

All eight microsatellite loci were moderately to highly polymorphic, with observed and expected heterozygosity (Table 2). All populations exhibited similar values of allelic richness, but the year 2002 showed the highest allelic richness and observed heterozygosity value ($p < 0.05$). Several significant deviations from Hardy-Weinberg expectations were observed, mainly due to loci ANG 075 and ARO 095. These two loci are suspected to exhibit null alleles, but they did not influence estimates of genetic differentiation when Dannewitz *et al.* (2005) repeated analyses with or without them.

Genetic differentiation and cluster analysis – Overall genetic differentiation at allozymes was low ($F_{ST} = 0.0099$), but highly significant ($p < 0.001$). We partitioned genetic variation among geographical locations, among temporal samples within locations, and within samples. Most of the genetic variation was present within samples (> 99.5%), the remaining variability was distributed among temporal samples within site ($p < 0.01$), while the between site component

exhibited a negative value. Nevertheless, the power of this approach was low, because of a low number of groups and very similar and low levels of differentiation within and between sites, prompting for additional analyses. Pairwise genetic differentiation estimates (F_{ST}) were grouped into within-year between sample comparison and a between-year within-sites comparison (Figure 2a, b). Temporal genetic differentiation within sites was significantly higher in the Western French population than in the others (ANOVA; F[5,10] = 5.26, p = 0.01). Southern France showed the lowest temporal differentiation (F_{ST} = 0). Genetic differentiation between sampling sites was similar in 2001 and 2003, but tended to be lower in 2002 (p = 0.7). A neighbour-joining dendrogram based on pairwise Cavalli-Sforza & Edwards (1967) genetic distances showed neither geographic clustering, nor temporal homogeneity (data not shown). All D_{CE} distance values between consecutive cohorts were nevertheless significant (p < 0.05).

Overall genetic differentiation at microsatellites was low (F_{ST} = 0.0024), but highly significant (p <0.001). When partitioning genetic variation among geographical locations, among temporal samples within locations and within samples, again most genetic variation was present within samples (> 99.5%). The remaining variability was distributed among temporal samples within sites (p < 0.01); the between site component exhibited a negative value.

Table 2: Genetic diversity estimates of each *Anguilla anguilla* cohort. Expected unbiased heterozygosity (H_e), observed heterozygosity (H_O) and allelic richness (R) are presented. For sample abbreviations, see Table 1.

Sample	Allozymes			Microsatellites		
	H_e	H_o	R	H_e	H_o	R
IC01g	0.1647 (0.2110)	0.1807 (0.2328)	2.49	0.7917 (0.2031)	0.7010 (0.2035)	12.54
IC02g	0.1814 (0.2001)	0.1722 (0.1906)	2.74	/	/	/
IC03g	0.2006 (0.2259)	0.2124 (0.2400)	3.01	0.7787 (0.1996)	0.6013 (0.2151)	12.16
IR01g	0.1856 (0.1992)	0.1694 (0.1711)	2.88	0.7549 (0.2716)	0.6883 (0.2553)	11.30
IR02g	0.1927 (0.2149)	0.1903 (0.2197)	3.14	0.7701 (0.2417)	0.7244 (0.2234)	11.90
IR03g	0.2124 (0.2108)	0.2125 (0.2292)	3.20	0.7768 (0.2136)	0.6470 (0.2258)	11.37
NL01g	0.1931 (0.1994)	0.2000 (0.2174)	3.03	0.7749 (0.2311)	0.6607 (0.2179)	11.72
NL02g	0.1846 (0.2161)	0.1777 (0.2193)	2.93	0.7724 (0.2468)	0.7144 (0.2274)	12.45
NL03g	0.1876 (0.2139)	0.1972 (0.2351)	2.97	0.7804 (0.2207)	0.7005 (0.2342)	11.65
FR01g	0.1685 (0.1658)	0.1653 (0.1746)	2.60	0.7799 (0.2133)	0.7248 (0.1882)	11.86
FR02g	0.1692 (0.2217)	0.1694 (0.2275)	2.84	0.7827 (0.2399)	0.6859 (0.2319)	12.37
FR03g	0.1666 (0.1861)	0.1569 (0.1721)	2.95	0.7689 (0.2206)	0.6255 (0.2307)	11.48
TV01g	0.1879 (0.2111)	0.1986 (0.2306)	3.05	0.7701 (0.2365)	0.6485 (0.2553)	11.86
TV02g	0.1949 (0.2086)	0.1833 (0.1960)	3.02	0.7778 (0.2397)	0.6976 (0.2371)	12.15
TV03g	0.2015 (0.2100)	0.1833 (0.1932)	2.80	0.7784 (0.2154)	0.6576 (0.1953)	12.71
MA99g	0.1769 (0.1950)	0.1787 (0.1975)	2.84	0.7823 (0.2246)	0.7277 (0.2205)	12.50
MA01g	0.1962 (0.2001)	0.1917 (0.1933)	3.12	0.7842 (0.1931)	0.6900 (0.1937)	11.58

Similar to allozymes, due to a low number of groups and very similar low differentiation within and between sites, the power of the analysis was low. Pairwise genetic differentiation estimates were grouped into a within-year between-sample comparison and a between-year within-site comparison (Figure 2c, d). Temporal genetic differentiation within sites was the highest in The Netherlands and the lowest in Ireland, but non significant ($p = 0.7$). Genetic differentiation between sampling sites was similar in 2001 and 2003, but significantly lower in 2002 ($p < 0.05$). The differences in distribution of pairwise $F_{ST}/1\text{-}F_{ST}$ values between groups differing 0, 1 and 2 years was significant (KW-H (2.105) = 7.25, $p < 0.05$), with higher differentiation between more samples that are more distant in time. A neighbour-joining dendrogram based on pairwise Cavalli-Sforza & Edwards (1967) genetic distances showed no geographic clustering, nor temporal homogeneity (data not shown). All D_{CE} distance values between consecutive cohorts were significant ($p < 0.05$).

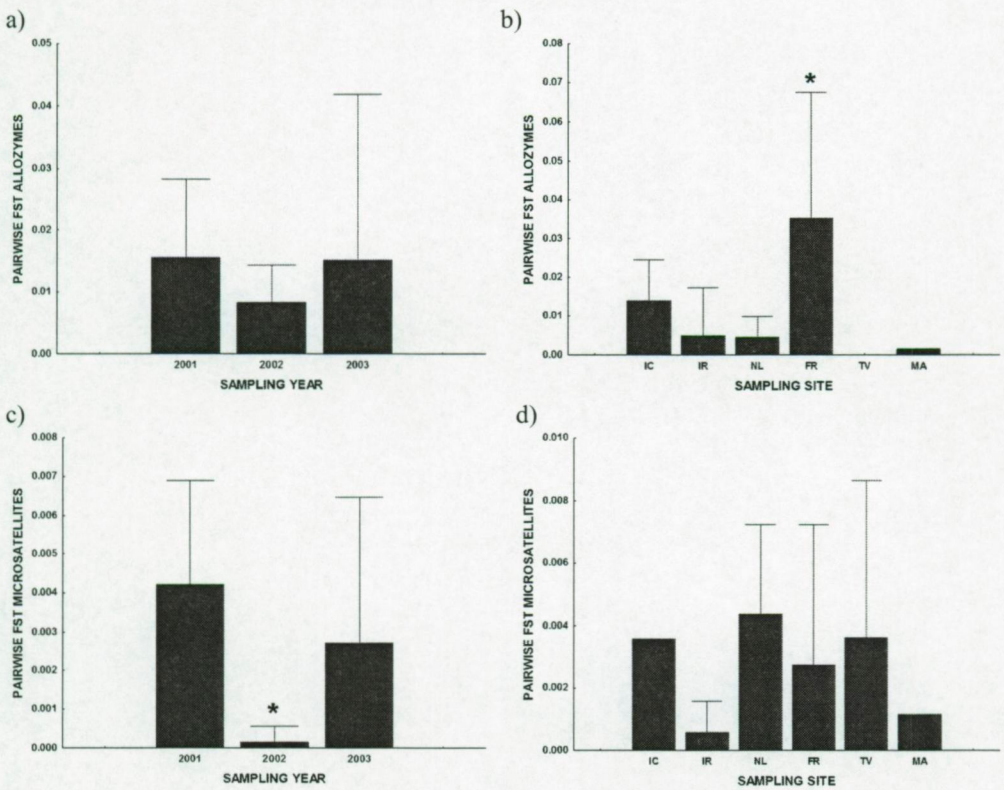


Figure 2 : Intra-annual (geographical) and inter-annual (temporal) genetic differentiation plot for (a, b) allozymes and microsatellites (c, d). Vertical bars represent the 95 % confidence interval. * = $p < 0.05$. For sample abbreviations, see Table 1.

Correlation between geographical, temporal and genetic distance – In the case of allozymes, there was a significant negative correlation between coastal distance and $F_{ST}/1\text{-}F_{ST}$ using a Mantel test (r = -0.198, p = 0.02). A temporal Mantel test did not reveal any significant correlation between differences in recruitment (years or days) and $F_{ST}/1\text{-}F_{ST}$ (r = 0.07, p > 0.05; r = 0.005, p > 0.05, respectively). In the case of microsatellites, there was a positive but non-significant relation between coastal distance and $F_{ST}/1\text{-}F_{ST}$ using a Mantel test (r = 0.13, p > 0.05) (Figure 3a). A temporal Mantel test revealed a significant positive correlation between differences in time of recruitment in years (r = 0.186, p = 0.03, Figure 3b), and a strong positive relationship, when expressing recruitment in days (r = 0.297, p = 0.004, Figure 3c). The geographical and temporal distances between samples were partitioned using a partial Mantel test. It revealed that the coefficient of correlation was twice as high using temporal distance than geographical distance (Table 3).

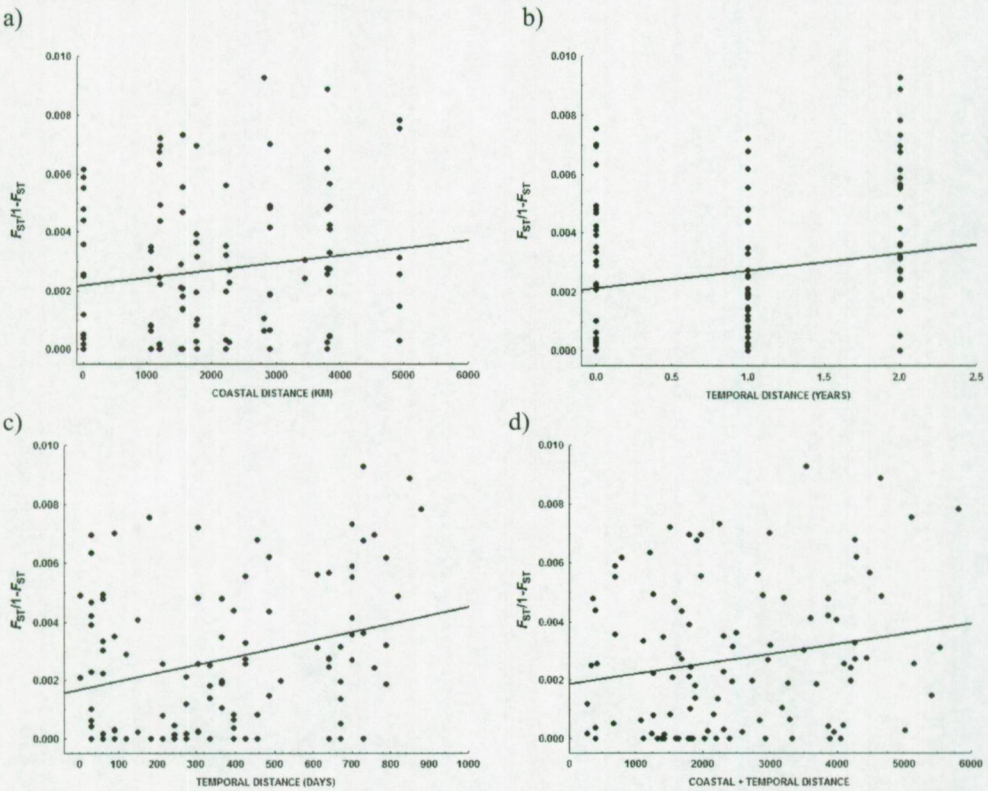


Figure 3 : *Anguilla anguilla* : Mantel tests : Correlation between genetic differentiation ($F_{ST}/1\text{-}F_{ST}$) at microsatellites and (a) coastal distance between sites (r = 0.13, p > 0.05); (b) temporal distance in years between cohorts (r = 0.186, p < 0.05); (c) temporal distance in days between cohorts (r = 0.297, p < 0.01) and (d) a combined temporal-geographical distance (r = 0.195, p < 0.05).

The percentage of variation explained by the model (R^2) was 13 %, corresponding to 10.46 % of the variation explained by temporal (p = 0.0015) and 2.28% of the variation explained by geographical (p = 0.12) distance. Combining both distances in one model does not yield a much higher R^2 and correlation coefficient (13 % versus 12.74% separately) (r = 0.195, p < 0.05, Figure 3d). To test for the impact of single populations (e.g. due to possible hybrids in Iceland and Morocco; Maes *et al.* 2005, in prep) or the influence of the highest temporally differentiated populations (FR and NL), we repeated the temporal Mantel test by first sequentially removing IC, MA and the Mediterranean population TV. We then removed FR and NL in the central zone. The correlation coefficients remained roughly constant, although significance decreased after removing sites (Table 3). When partitioning daily distances into three classes (corresponding to 1, 2 or 3 years difference), the correlation coefficient within class increased from r = -0.21, p = 0.13; r = 0.21, p = 0.33 to r = 0.445, p = 0.01. After restricting the temporal Mantel analysis within each year (seasonal), no significant correlation was visible anymore between temporal distance in days and genetic distance (2001: r = 0.27, p = 0.27; 2003: r = -0.41, p = 0.94).

Table 3: Relationship between geographical, temporal (years and days between cohorts) and genetic ($F_{ST}/1-F_{ST}$) distance in European glass eel (*Anguilla anguilla*) cohorts. Single and partial Mantel tests are listed to discriminate between the influence of geographical and temporal distances on genetic differentiation. For country abbreviations, see Table 1.

Marker	Single Mantel tests	R	p-value
Allozymes	Coastal Distance	-0.198	0.02
	Yearly Distance	0.070	0.2
	Daily Distance	0.005	0.45
Microsatellites	Coastal Distance	0.130	0.17
	Yearly Distance	0.186	0.03
	Daily Distance	0.297	0.004
	Daily Distance -no IC	0.339	0.005
	Daily Distance -no IC+MA	0.317	0.014
	Daily Distance -no IC+MA+TV	0.272	0.06
	Daily Distance -no FR	0.345	0.012
	Daily Distance -no FR+NL	0.272	0.08

	Partial Mantel tests	R	R^2	p-value
Microsatellites	Coastal Distance	0.161	2.28	0.12
	Daily Distance	0.323	10.46	0.0015
	Total		13.04	

Pairwise relatedness within and between cohorts – Because allozymes in this study showed a fairly high proportion of low polymorphic loci and relatedness is inversely correlated with the number of rare or low frequency alleles, average relatedness may better be calculated using highly variable microsatellite loci. In general, most populations were composed of outbred and negatively related individuals (negative r_{xy} values), but also related ones ($r_{xy} > 0.5$) (Table 4). The individuals within the samples NL03g, FR02g and FR03g were more related than by chance ($p < 0.05$) (Table 4). An analysis of variance showed that mean pairwise relatedness differed significantly between yearly cohorts of IR and NL ($p < 0.05$). After pooling all temporal samples by site, the IR samples ($p < 0.05$) and the FR samples ($p < 0.01$) were more related than by chance (Table 4). NL and MA exhibited both the highest as the lowest r_{xy} values (MAX-MIN, Table 4).

Table 4: *Anguilla anguilla*. Average pairwise genetic relatedness (r_{xy}) following Queller & Goodnight (1989). Values were calculated between all individuals within each sample, within each geographical location by pooling temporal samples and between sites within a year (assuming one spawning population). MIN: minimum r_{xy} value, MAX: maximum r_{xy} value and S.D. :standard deviation. * = $p < 0.05$; ** = $p < 0.01$.

Year	Sample	Mean r_{xy}	S.D.	MIN r_{xy}	MAX r_{xy}
2001	IC01G	-0.0081	0.102	-0.37	0.7
2003	IC03G	-0.0113	0.122	-0.48	0.88
2001	IR01G	-0.0044	0.108	-0.52	0.6
2002	IR02G	-0.0031	0.100	-0.42	0.75
2003	IR03G	-0.0123	0.103	-0.5	0.59
2001	NL01G	0.0137	0.225	-0.86	1
2002	NL02G	-0.0095	0.110	-0.78	1
2003	NL03G	-0.0055*	0.099	-0.47	0.52
2001	FR01G	-0.0040	0.106	-0.39	0.5
2002	FR02G	-0.0098*	0.109	-0.59	0.71
2003	FR03G	-0.0090*	0.137	-0.62	0.65
2001	TV01G	-0.0033	0.104	-0.41	0.74
2002	TV02G	-0.0051	0.103	-0.48	0.75
2003	TV03G	-0.0028	0.111	-0.37	0.77
1999	MA99G	-0.0077	0.118	-0.69	1
2001	MA01G	-0.0056	0.116	-0.74	0.71
ALL	IC	-0.0007	0.114	-0.5	0.89
ALL	IR	-0.0015*	0.104	-0.63	0.8
ALL	NL	0.0054	0.135	-0.83	1
ALL	FR	0.0004**	0.119	-0.56	0.81
ALL	TV	-0.0018	0.103	-0.45	0.85
ALL	MA	-0.0031	0.116	-0.75	1
2001	ALL SAMPLES	0.0060*	0.129	-0.89	1
2002	ALL SAMPLES	-0.0004**	0.109	-0.74	1
2003	ALL SAMPLES	0.0004**	0.113	-0.57	1

The average relatedness was highest in NL, differing significantly from all other sites ($p < 0.01$), and lowest in MA. When considering the whole year cohort, there was a non-random distribution of individual relatedness values within the three years ($p < 0.05$), and relatedness also differed between years ($p < 0.05$). The average relatedness was highest within a year (locations pooled), then within a pooled geographical site and the lowest within cohorts, which indicates that genetically distinct groups were pooled. In the year 2001 average relatedness was highest, while in 2002 it was lowest ($p < 0.05$).

DISCUSSION

Evidence for Isolation-by-Time within the European eel

The fundamental result of this study is the strong evidence for temporal partitioning of spawning cohorts following an Isolation-by-Time (IBT) model. We observed a highly significant correlation between genetic distance and temporal distance in days between recruiting eel cohorts, irrespective of geographical location, pointing to a patterned IBT effect. Populations situated in the central distribution zone (The Netherlands and Western France) exhibited the strongest temporal differentiation between cohorts and genetic differentiation was highest between glass eel cohorts differing two years in recruitment. Average relatedness also exhibited a non-random pattern within years and within the samples, especially in The Netherlands and Western France.

Isolation-by-Time has never been detected or directly tested in other marine organisms. Ruzzante *et al.* (1996) found evidence for heterogeneous larval cohorts in cod *Gadus morhua* collected over a 3-week period. It was attributed to a sweepstakes event, although the correlation between genetic distance and spawning time was not tested. Similarly, no IBT was tested in studies analysing successive groups of spawning adults in cod (Ruzzante *et al.*, 1999; Beacham *et al.*, 2002), red drum (*Sciaenops ocellatus*, Chapman *et al.*, 2002), plaice (*Pleuronectes platessa*, Hoarau *et al.*, 2002) and herring (*Clupea harengus*, McPherson *et al.*, 2003). Most studies detected a temporal Wahlund effect within spawning sites, pointing to a succession of different spawning aggregations separated in time, which is concordant with Sinclair's (1988) member-vagrant hypothesis.

The IBT pattern reported here has never been observed before in eel, although several studies have shown patterns of clinal variation and Isolation-by-distance (IBD) in American eel (Koehn & Williams, 1978), Japanese eel (Chan *et al.*, 1997) and European eel (Wirth & Bernatchez, 2001; Maes & Volckaert, 2002). Allozymatic clinal variation in these studies was

mostly ascribed to post-larval selection, while mitochondrial and microsatellite DNA studies proposed a subtle spatial isolation or temporal delay in spawning times. A partial Mantel test successfully partitioned the genetic variation in a very subtle geographical component and a stronger temporal component. To detect IBT, temporal replication has to be performed within one single life stage, to enable the use of a continuous timescale without a reproductive event in between. The mixing of juveniles and adults consisting of several year classes may blur a signal of IBT, unless offspring of spawning cohorts can be sampled after reproduction. We believe that the IBD pattern observed in former studies might be either due to introgressive hybridisation of American eel genomes in Northern European eel populations (Maes *et al.*, 2005, in prep; Wirth *et al.*, 2005, in prep) or to the sampling of temporally separated individuals at different locations (IBT). Considering the temporal delay in the migration of adult eels, the reported IBD pattern can then be equivalent to an IBT pattern modulated by a substantial noise component (Waples, 1998) originating from the mixing of various age classes in adults (Tesch, 2003).

Absence of a seasonal IBT pattern: genetic patchiness within cohorts

Considering the theoretical expectations of an IBT model, populations are composed of individuals that reproduce at different times within a reproductive season, which are often heritable. Limited gene flow can arise between early and late spawners owing to heritable reproductive times. The heritable component of reproduction remains unknown in eel, although the timing of the spawning migration is synchronized with oceanic factors influencing the reproductive success (Okamura *et al.*, 2002). In our study, when testing for IBT within cohorts: (1) temporal distance showed no correlation with genetic distance, and, (2) samples from comparable sampling dates did not clustered together in the neighbour-joining dendrogram. The absence of a consistent seasonal IBT pattern suggests that there is no heritable component in the seasonal spawning migration of eel. Nevertheless, it must be taken into account that our study included samples mostly from the main arrival peak of glass eels. Thus, our intra-annual sampling might not have covered early and late larval cohorts. On the other hand, heritable reproductive times might be blurred by a large variance in age at maturity in eels, which is highly dependent on environmental factors and can range from 6 to 50 years (Poole & Reynolds, 1996; Tesch, 2003).

Examination of genetic differentiation within cohorts suggest a pattern of genetic patchiness among samples. Although the pattern of IBT was highly significant, only 13 % of

the variance in allele frequencies could be explained by temporal and geographical distance. The inter-cohort component of genetic differentiation was low (F_{ST} allozymes = 0.9 %; F_{ST} microsatellites = 0.2%), but highly significant and similar to values reported in studies analysing adults and juveniles (Wirth & Bernatchez, 2001; Maes & Volckaert, 2002; Dannewitz *et al.*, 2005). At small temporal scales, genetic patchiness may play a substantial role, given the various "high" F_{ST} values at small temporal distances (differing 0 to 300 days) (Figure 3). Additionally, there was no obvious spatio-temporal clustering of cohorts. If temporal differentiation were strong, we would expect a clustering of samples by year and not by geographical site. Stochastic fluctuations in reproductive success clearly interfere with a discrete temporal differentiation, indicating the continuous nature of IBT in eels over the years. Geographical differentiation was much lower in the year 2002, compared to other years, an indication that variance in reproductive success may be dependent on yearly fluctuating environmental conditions.

The influence of genetic patchiness can be detected within the samples of Western France and The Netherlands. The low genetic variability and high temporal differentiation in Western France are conform to the genetic sweepstakes hypothesis, predicting a decrease in genetic variability and high variance in allele frequencies if strong genetic drift acts on spawning populations (Hedgecock, 1994). The Gulf of Biscay is the main arrival region for glass eels (Tesch, 2003) and mixing of recruiting glass eel cohorts may occur less in this area, judged from the morphometric analyses. The shelf edge (200m-isobath) line in this region is close to the coast, which enables the direct ascent of glass eels to the rivers (Bertin, 1956), resulting in the observed signal of temporal differentiation. In the case of The Netherlands, samples are more likely to be composed of intermingling glass eel groups, as they usually wait three months in the North Sea before ascending rivers (Boëtius & Boëtius, 1989).

Oceanic influence on morphometric and genetic traits

Marine taxa are strongly influenced by climatic and oceanic conditions in their early life stages (Attrill & Power, 2002; Beaugrand *et al.*, 2003). Morphometric variables should mirror oceanic conditions experienced by larvae. Our morphometric analyses showed strong spatio-temporal differences in length, weight and condition between cohorts within a year and between years. Such differences were the strongest in the central distribution zone of eel (The Netherlands and Western France). Interestingly, the condition index showed a very consistent pattern between sites among years, irrespective of seasonal differences (Figure 1). Over the

years, length increased, weight fluctuated and condition decreased. Earlier studies on morphometry of glass eel reported similar trend, namely an increase in length during the last years (Dekker, 2003), a strong dependence of weight on the trophic capacity of the oceans (Desaunay & Guérault, 1997) and a lower condition in northern regions (Boëtius & Boëtius, 1989). Oceanic and climatic conditions (such as the North Atlantic Oscillation) affect larval development, growth and survival, by influencing food supplies in retention zones and current patterns during migration (Agostini & Bakun, 2002; Largier, 2003). These factors could explain the lower recruitment and lower length and weight of glass eels observed during the last decennia (Knights, 2003). Condition, however, was more site-specific and was strongly correlated to the distance to the 200 m-isobath, a distance determining the length of the starvation period in glass eels (Bertin, 1956). Boëtius and Boëtius (1989) showed that eel larvae metamorphosing in Northern Europe do not ascend directly to the rivers but are forced to stay offshore, where they are starving. In The Netherlands, this period has been estimated to last about three months (Desaunay and Guérault, 1997), which may explain the lower condition of the samples from The Netherlands. Although morphometric variables fluctuate over time, they are strongly correlated with the winter NAO index of the year before, pointing to the importance of integrating an oceanic component in eel management strategies (Knights, 2003).

Although the genetic variability within glass eel cohorts was very low among sites and years, and in accordance to previous genetic studies on eel (Daemen *et al.*, 2001; Wirth & Bernatchez, 2001; Maes & Volckaert, 2002; Dannewitz *et al.*, 2005), we found several indications for environmental dependence of genetic variation. A higher allozymatic polymorphism at lower latitudes suggests that many rare alleles are maintained in the southern distribution area. This is in contrast with a recent mitochondrial DNA study, reporting a higher number of haplotypes in Northern samples (Daemen *et al.*, 2001). Populations in Southern Europe exhibited a lower inter-annual differentiation, pointing to a more stable and successful spawning period or weaker post-larval selection. There were also several indications for a relation between oceanic conditions (using the NAO index as proxy) and genetic estimators. First, a higher allelic richness was detected in years with negative NAO values associated with good oceanic conditions. The opposite was visible in years of positive NAO values. Secondly, the highest genetic differentiation between cohorts was observed in years with a positive NAO index (2000-2001), which may be linked to less successfully reproducing parents (Hedgecock, 1994). Finally, if cohorts associated with poor feeding conditions were constituted by a lower number of families, a positive correlation

between the NAO index and relatedness would be expected. In our study, a negative trend was observed, such that individuals were more related in cohorts associated with poor feeding conditions. Further validation is needed to confirm the synchronicity of ocean climate, feeding conditions and recruitment abundance.

Large variance in reproductive success in eel triggers Isolation-by-Time

In contrast with the lack of a seasonal IBT pattern, a clear IBT pattern was observed between years, namely between cohorts differing 1.5 to 2.5 years. Since a heritable component inducing IBT remains questionable, a transient pattern of IBT can arise mainly under the influence of environmental factors. We suggest a possible scenario for the spatio-temporal genetic structure of the European eel based on its catadromous life-strategy, which is affected by a long migration loop and oceanic factors that lead to a large variance in parental contribution. Migrating silver eels start their spawning migration at different times through the year, usually between September and November (Tesch, 2003). Due to the distance to the Sargasso Sea, differential departure times added up with differential migration distances induce a protracted spawning window in the Sargasso Sea. Gene flow will be minimal between cohorts that differ the most in spawning time. The Sargasso Sea might consist of one or several distinct retention zones for larvae, where several spawning groups spawn partially connected by gene flow in time and space (Sinclair, 1988). Mixing may be largely restricted to neighbouring spawning cohorts, producing a continuously increasing genetic distance between successive cohorts (IBT), triggered by subtle adult genetic backgrounds or variance in reproductive success. If the traits influencing age at maturity and spawning time/place would be heritable, this pattern should be stable over time and independent of oceanic conditions. In our study, however, the lack of seasonal IBT pattern indicates a large scale difference in spawning aggregation between years, most likely due to stochastic effects. Once larvae hatch, strong selection can act on each larval pool, inducing high mortality and reducing the reproductive success of many spawning cohorts even more (Strathmann, 1993; Beaugrand *et al.*, 2003). The resulting genetic stochasticity may produce high variance in allele frequencies between adjacent larval cohorts. In years with above average climatic conditions, more unrelated individuals would spawn successfully, resulting in a lower inter-cohort genetic differentiation, while keeping more genetic variation within larval pools. This genetic patchiness is maintained up to the continental shelf, superimposed on the genetic signal of differential reproductive timing in the glass eel genomes.

In summary, we detected a double pattern of variance in genetic composition. (1) A broad scale Isolation-by-Time pattern of spawning cohorts separated by 2-3 years, possibly as a consequence of the long migration loop in anguillids and strong variance in annual adult reproductive success; (2) a smaller scale variance in reproductive success (genetic patchiness) among seasonally separated cohorts, most likely originating from variables driven by oceanic and climatic drivers. IBT on a broader scale and genetic patchiness on a local scale are not contradictory but a consequence of the catadromous-life strategy of the European eel, which is affected by a long migration loop and oceanic factors that lead to a large variance in parental contribution.

ACKNOWLEDGMENTS

We thank the following people for their help in obtaining samples: B. Jonsson, R. Poole, W. Dekker, J. Van Willigen, P. Elie, A. Crivelli and A. Yahyaoui. Helpful comments on earlier versions were given by T. Wirth, E. Nielsen, P. Galbusera, L. De Meester and F. Ollevier. G.E. Maes received a PhD fellowship from the I.W.T. (Institute for the Promotion of Innovation by Science and Technology in Flanders). J.M. Pujolar received a postdoctoral fellowship from the Ministerio de Educación, Cultura y Deporte (Spain). Both authors were supported by the EU contract EELREP (Q5RS-2001-01836).

PART C:

Relationship between genetic variability and fitness in the European eel (*Anguilla anguilla* L.)

CHAPTER VI:

The catadromous European eel *Anguilla anguilla* (L.) as a model for freshwater evolutionary ecotoxicology: relationship between heavy metal bioaccumulation, condition and genetic variability

Maes G.E., Raeymaekers J.A.M., Pampoulie C., Seynaeve A., Goemans G., Belpaire C. and Volckaert F.A.M.

SUMMARY

Understanding the effects of pollutants on the genome is of crucial importance to preserve the evolutionary potential of endangered natural populations. The highly vagile European eel (*Anguilla anguilla* L.) has suffered a dramatic decline in recruitment since two decades, urging for a better understanding of the genetic impact of pollution. Its catadromous life history constitutes a model to assess local selection of pollutants on condition and genetic variability, as juveniles recruit in European rivers without appreciable pollution load or interfering genetic background. Because of its high fat content and local benthic feeding behaviour, the feeding stage is considered extremely prone to the bioaccumulation of pollutants. We studied the relationship between heavy metal bioaccumulation, fitness (condition) and genetic variability in the European eel. The muscle tissues of 78 sub-adult eels, originating from three Belgian river basins (Scheldt, Meuse and Yser), were examined for nine heavy metal pollutants (Hg, Cd, Pb, Cu, Zn, Ni, Cr, As and Se), while in total 123 individuals were genotyped at 12 allozyme and 8 microsatellite loci. A significant negative correlation between heavy metal pollution load and condition was observed, suggesting an impact of pollution on the health of sub-adult eels. In general, we observed a reduced genetic variability in strongly polluted eels, as well as a negative correlation between level of bioaccumulation and allozymatic multi-locus heterozygosity (MLH). Microsatellite genetic variability did not show any pollution related differences, suggesting a differential response at metabolic enzymes and possibly direct overdominance of heterozygous individuals.

Keywords: evolutionary toxicology; multi-locus heterozygosity; nuclear markers; river pollution; selection.

This chapter is published in Aquatic Toxicology 73: 99-114

INTRODUCTION

Evidence from animal and plant populations indicates that allozymatic polymorphism and heterozygosity might be linked to environmental heterogeneity and stress (Nevo *et al.*, 1986; Ben-Shlomo & Nevo, 1988; Chagnon & Guttman, 1989; Gillespie & Guttman, 1989; Stanton *et al.*, 2000; Nevo, 2001). Understanding the effects of pollutants on the genome is of crucial importance to preserve the evolutionary potential of endangered natural populations, as a high genetic diversity provides a population the potential to adapt to selective forces (Gillespie & Guttman, 1989). Under natural conditions (e.g. absence of anthropogenic influences), allelic frequencies within a population fluctuate with time according to stochastic processes (drift), migration and/or environmental selection pressures (such as climate or habitat changes), while maintaining polymorphism. However, severe perturbations on a short temporal scale, such as man-induced pollution and harvesting, may lower the condition and genetic variability, reducing the viability (fitness) of natural populations. Hence the susceptibility to additional environmental stress increases, weakening the survival of the species (Thorpe *et al.*, 1981; Leary *et al.*, 1987; Stanton *et al.*, 2000). The importance of genetic variation to survive anthropogenic environmental changes relates to factors such as resistance to heat stress (mummichog, *Fundulus heteroclitus*; Powers *et al.*, 1991), oil pollution (mussels, *Mytilus edulis*; Fevolden & Garner, 1986) and radiation (central stoneroller, *Campostoma anomalum*; Gillespie & Guttman, 1989).

There are four ways in which toxicants may affect the genetic variability in a population (Van Straalen, 1999; Van Straalen & Timmermans, 2002): (1) some toxicants are mutagenic, increasing directly the mutation rate; (2) they may indirectly affect the mutation rate by affecting DNA repair mechanisms; (3) they may favour more tolerant genotypes than others and change the genetic composition of the population towards a higher mean tolerance; and (4) they may cause bottlenecks or alter migration. The first two mechanisms will increase genetic diversity, while the two latter ones will decrease it, possibly exhausting genetic variation in natural populations. This process is referred as "genetic erosion" (Van Straalen & Timmermans, 2002).

The impact of pollutants or toxicants, such as heavy metals, pesticides or industrial waste, on the genetic diversity and structure of natural populations relates to a reduced genetic variability in polluted populations, genotype-specific survivorship and subsequent shift in the distribution of tolerant genotypes without net loss of diversity, or significant correlations between pollutants and allele frequencies (Hvilsom, 1983; Fevolden & Garner; 1986; Klerks

& Weis, 1987; Patarnello & Battaglia., 1992; Posthuma & Van Straalen, 1993). Heavy metal pollutants seem to strongly affect allelic selection or allele frequency shifts at polymorphic loci (Hvilsom, 1983; Ben-Shlomo & Nevo, 1988; Chagnon & Guttman, 1989; Frati *et al.*, 1992). Most of these studies focused on well-defined populations, with low dispersal capability and reproducing locally. Organisms with a catadromous life history (i.e. spawning at sea, feeding in rivers and lakes) are expected to reflect local pollutants impact faithfully, as somatic and population genetic comparisons can be made after dispersal without worrying about different genetic background, parental influence or larval pollution load. Species with a high effective population size (mostly marine) generally exhibit high levels of heterozygosity and are expected to be more resistant to pollution; multi-locus heterozygotes often show an increased fitness over homozygotes (Nevo *et al.*, 1986; David, 1998). The question remains whether the effect of pollutants can also be measured on condition and genetic variability in highly vagile species.

There are few analyses of the relationship between the bioaccumulation of contaminants and genetic diversity in natural populations (Van Straalen, 1999). An important aspect when quantifying contaminant pressure is not only the exposure concentration but also the actual uptake of the contaminant in the body, namely the level of bioaccumulation (Van der Oost *et al.*, 2003). Concentrations of environmental pollutants do not always reflect the actual level of contamination of the individuals; lab based experimental studies often use higher concentrations than present in the natural habitat (Newman & Jagoe, 1998, but see Belfiore & Anderson, 2001). Hence, a combination of experimental and field-based studies remains ideal to encompass both molecular and population-genetic influences of environmental contaminants (Bickham *et al.*, 2000).

The organism of interest in this study is the European eel (*Anguilla anguilla* L., Anguillidae, Teleostei), a marine fish spending most of its lifetime in European freshwater rivers, lagoons or lakes, but spawning in the Sargasso Sea in the South-Western Atlantic (Tesch, 2003). Leptocephali larvae migrate along the Gulf Stream and North Atlantic Drift to reach the European continent, enter the rivers as glass eels, feed at least for 6 (males) to 8 years (females) until their spawning migration as silver eels (Tesch, 2003). Its catadromous life history constitutes a model to assess local selection of pollutants on condition and genetic variability, as juveniles recruit without appreciable pollution load or interfering genetic background. Despite extensive spawning migrations, the feeding stage (yellow eel) seems relatively sedentary (Tesch, 2003). In fact, because of its high fat content and local benthic

feeding behaviour, the sub-adult stage is considered extremely prone to the bioaccumulation of pollutants (Linde *et al.*, 1996; Roche *et al.*, 2003).

The European eel stock is declining rapidly and is now at its minimum since 1970, probably due to climate/current changes, but compounded by anthropogenic changes like habitat destruction, migration barriers, overfishing and pollution (Dekker, 2000, Feunteun, 2002). The impact of pollutants in eel is known (see Robinet & Feunteun, 2002 for a review), but it is unclear to what extent the reproductive potential is affected and whether strong differential selection may alter the genetic composition of resident freshwater populations before the spawning migration.

Although there is firm proof that higher pollution levels decrease fitness traits, the impact of genetic variability in a catadromous organism like eel to withstand environmental stress is not known. The objectives of this study were threefold: (1) we quantified the relationship between heavy metal bioaccumulation and fitness (condition) of yellow eels from three Belgian river basins, assuming that a high environmental load is reflected in the biota. (2) We tested the hypothesis of equal post settlement allozymatic and microsatellite genetic constitution among basins and among *post-hoc* defined groups exhibiting various levels of heavy metal bioaccumulation. (3) We tested whether there is a correlation between multi-locus heterozygosity (MLH) at both genetic markers, the individual level of bioaccumulation and condition. If contaminants cause selective mortality of individuals with specific genomes, then allele and genotype frequencies should differ between populations from impacted sites (lower genetic variability) and those from not or less impacted sites (Gillespie & Guttman, 1989). A positive correlation between MLH and condition indices or negative correlation with level of bioaccumulation, would suggest a higher fitness (less contamination) for more heterozygous individuals (heterosis) (Nevo *et al.*, 1986).

MATERIAL AND METHODS

Samples – A total of sixteen sites were sampled in three river basins (Figure 1). Approximately ten sub-adult freshwater eels (yellow eel stage) were collected either by electro-fishing or with fyke nets at each site during the year 2000 (N= 123). The sampling was part of an extensive eel pollution-monitoring network for Flanders in 2000 (Goemans *et al.*, 2003). To detect inter-basin differences and to improve the statistical power of the analyses (especially by avoiding low sampling bias in genetic variability estimates), we initially grouped samples by river basin. The first set of samples originated from the Lower

Scheldt (S1, S2, S3, S4, S5, S6 and S7), the second set was sampled in the river Meuse (M1, M2, M3 and M4), while the third set was sampled in the river Yser (Y1, Y2, Y3, Y4 and Y5) (Figure 1). Eels were kept alive in oxygenated tanks for maximally three days and processed in the laboratory. Standard length (L), body weight (W_B) and liver weight (W_L) were determined for each individual. Samples from muscle and liver tissues were collected for ecotoxicological (heavy metal concentration) and genetic (allozymes and microsatellites) analyses.

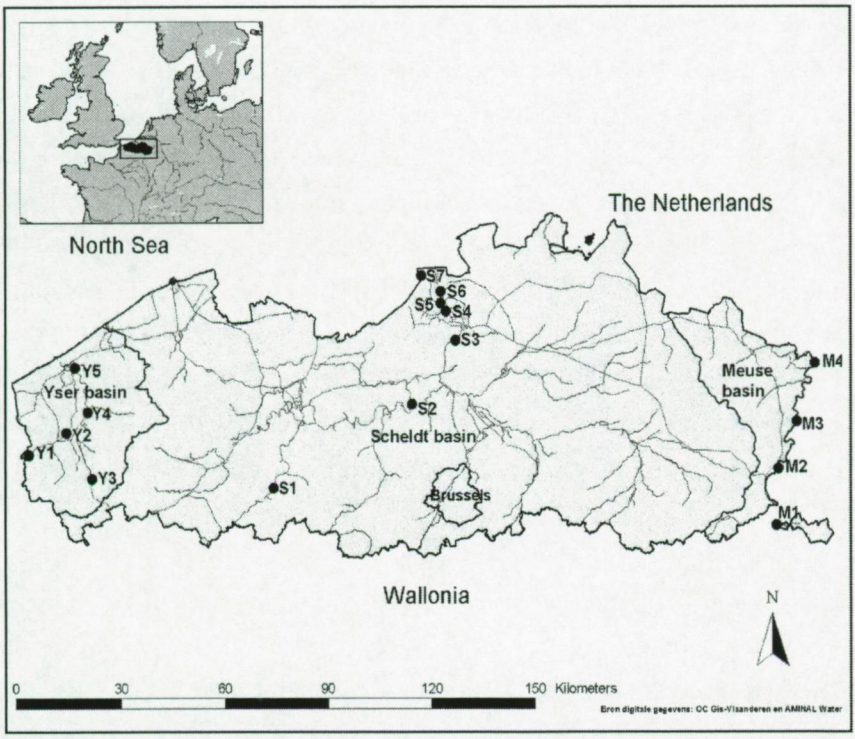


Figure 1: *Anguilla anguilla*: Belgium (Flanders) with sampling sites along three river basins; Yser: Y1, Y2, Y3, Y4, Y5; Scheldt: S1, S2, S3, S4, S5, S6, S7; Meuse: M1, M2, M3, M4.

Heavy metal load measurements and analysis – A sample of about 3-5 g of muscle tissue was removed, labelled and frozen at –20°C before analysis. A total of nine heavy metal concentrations were measured for three to six eels per sampling site (N= 78) by ICP-OES (Inductive Coupled Plasma-Optical Emission Spectrometry) for Cr, Ni, Cu, Zn, Cd and Pb. Quantification of As and Se was performed by GF-AAS (Graphite Furnace Atomic Absorption Spectrometry) according to standard procedures (Skoog, 1997, Goemans *et al.*,

2003). Concentrations were expressed in $\mu g.kg^{-1}$ (Hg, Cd, Pb, Ni, Cr, As and Se) or $mg.kg^{-1}$ (Cu and Zn) wet weight.

Allozyme electrophoresis – A total of 123 individuals (including the 78 individuals characterised for pollutants) were genotyped using Cellulose Acetate Gel Electrophoresis (CAGE, Harris & Hopkinson, 1976; Richardson, Baverstock & Adams, 1986). Electrophoresis and staining procedures followed Maes & Volckaert (2002). The most common allele was called '100' and other alleles were classified according to their relative mobility to allele '100' for the locus under study. The nomenclature used for enzymes followed Shaklee *et al.* (1990). Buffers used in the electrophoretic analyses were Tris-Glycine (TG) and Tris-Maleate (TM); both liver (L) and muscle (M) tissue were used. The following nine enzyme systems (coding for 16 loci) were scored: Alcohol dehydrogenase (*ADH-1**, *ADH-2**, EC 1.1.1.1, TG, L), Aspartate aminotransferase (*AAT-1**, *AAT-2**, EC 2.6.1.1, TM, L), Glucose-6-phosphate isomerase (*GPI-1**, *GPI-2**, EC 5.3.1.9, TG, M), Isocitrate dehydrogenase (*IDH-1**, *IDH-2**, EC 1.1.1.42, TM, L), L-lactate dehydrogenase (*LDH-A**, *LDH-B**, EC 1.1.1.27, TM, M), Malate dehydrogenase (*MDH-1**, *MDH-2**, EC 1.1.1.37, TM, L), Malic enzyme (*MEP-1**, *MEP-2**, EC 1.1.1.40, TM, L), Mannose-6-phosphate isomerase (*MPI*-1*, EC 5.3.1.8, TG, L) and Phospho-glucomutase (*PGM-1**, EC 5.4.2.2, TG, M). Twelve presumed polymorphic loci were scored to examine genetic diversity and genotype distribution.

DNA extraction and microsatellite amplification – Minute sections of tissue from ethanol preserved yellow eel fins (same individuals as allozymes, N = 123) were digested in a lysis buffer containing 200 µl 5% Chelex 100 solution (BioRad), 7 µl of 1M DTT (Dithiothreitol) solution pH 5.2 (diluted in 0.08M NaAc) and 10 µl Protein K solution ($10\ mg.ml^{-1}$) for at least 4 h at 56°C. After incubation at 100°C for 10 min, the samples were centrifuged at 13,000 rpm (10,000 g) for another 10 min; the supernatant was stored at –20°C for later analysis. Genotypes were examined at 8 dinucleotide repeat microsatellite loci: *AAN 01*, *AAN 02*, *AAN 05* (Daemen *et al.*, 2001); *ARO 095*, *ARO 054*, *ANG 151*, *ANG 114* and *ARO 121* (Wirth & Bernatchez, 2001). PCR reaction conditions were as follows: denaturation at 95°C for 3 min followed by a cycle of denaturation at 95°C for 35 s, annealing at 61°C (*AAN 01*, *AAN 02*) or 57°C (*AAN 05*) for 30 s and finally elongation at 72°C for 40 s. This cycle was repeated 30 (*AAN 01*, *AAN 02*) or 25 (*AAN 05*) times, after which an additional elongation of

10 min at 72°C was performed. Single PCR reactions consisted of 1 X PCR buffer (supplied with polymerase), $MgCl_2$ at a concentration of 1 mM (*AAN 02*, *AAN 05*) or 1.5 mM (*AAN 01*), 200 µM of dNTP, 0.4 µM of labeled forward and non-labeled reverse primer, 0.5 U of Goldstar *Taq* polymerase (Eurogentec, Seraing, Belgium) and 10-100 ng of gDNA. Double distilled water was added up to 10 µl. Loci *ARO 095*, *ARO 054*, *ANG 151*, *ANG 114* and *ARO 121* were run in a multiplex with the following PCR conditions: denaturation at 95°C for 5 min followed by a cycle of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and a final elongation at 72°C for 30 s. This cycle was repeated 25 times, after which an additional elongation of 8 min at 72°C was performed. Multiplex PCR reactions consisted of 1X PCR buffer (supplied with polymerase), $MgCl_2$ at a concentration of 1.5 mM, 80 µM of dNTP, on average 0.4 µM of fluorochrome labeled (IRD700 or 800, Westburg, The Netherlands) forward and non-labeled reverse primer, 0.5 U of Goldstar *Taq* polymerase and 10-100 ng of gDNA. Double dH_2O was added up to 25 µl. PCR products were run on a 5.5 % acrylamide 7 M urea sequencing gel using an automated sequencer (LICOR 4200). Along with the PCR products, a molecular ladder (Westburg) was run in order to quantify the allele sizes.

Analyses of condition and heavy metal data – To assess the relative condition of individuals under pollutant stress, two condition factors were used. (1) Ricker's (1975) condition index (CI) was calculated as $1000(W_B / L^b)$, where respectively L and W relate to standard length in millimetres and body weight in milligrams (King, 1995). (2) The hepato-somatic index (HSI) was calculated as $HSI = (W_L / W_B).100$, where W_L and W_B represent wet liver weight and wet body weight, respectively. The coefficient b in (1) was calculated as the slope from the Log W_B -Log L regression analysis for all three basins, as allometric growth was detected. We then assessed the relative condition of each individual from each basin using the formula (1). ANOVA tests were performed to compare the mean relative condition between basins. Since the liver is the major detoxification and lipid storage reserve organ, changes in weight of this organ will relate to detoxification and energy storage. Weight effects on HSI were removed from (2), followed by an ANOVA on the residuals of the weight-HSI regression for group comparison.

We used a Multivariate ANOVA on a set of seven heavy metals and univariate ANOVA's per metal followed by Tukey tests to detect the influence of basin on heavy metal load. We calculated a relative bioaccumulation index by dividing (standardizing) the individual concentration of heavy metal i (C_i) by the maximum observed concentration (C_{imax}) and

averaging over all metals, to relate heavy metal bioaccumulation to condition and genetic variability. Thus, the individual mean (multi-metal) bioaccumulation index (IMBI) was defined as:

$$\text{IMBI} = \left[\sum_{i=1}^{n}\left(C_i / C_{i\max}\right)\right] / n$$

with n = total number of metals, C_i = individual concentration of heavy metal i, $C_{i\max}$ = maximal observed concentration of heavy metal i and $0 < \text{IMBI} < 1$. To compare heavy metal bioaccumulation among basins, an ANOVA analysis was performed on the IMBI values, followed by post-hoc analyses (Tukey tests). We calculated Pearson's correlation coefficients between individual IMBI values and condition indices (CI and HSI) to assess pollutant impact on condition. All analyses were performed in STATISTICA version 6.0 (StatSoft).

Genetic data analyses – Allozymatic and microsatellite genetic diversity was evaluated based on genotype and allele frequencies, the level of polymorphism (P), observed and expected heterozygosity (H_O and H_E), total number of alleles and mean number of alleles per locus (MNA). Multi-locus heterozygosity (MLH) was calculated as the percentage heterozygous loci per individual (corrected for non scored loci). Homogeneity of allele frequencies among samples was tested with the program GENEPOP version 3.1d (Raymond & Rousset, 1995). Departures from Hardy-Weinberg (H&W) equilibrium were calculated as $D = (H_O - H_E) / H_E$ with GENEPOP version 3.1d (Raymond & Rousset, 1995) using the Markov chain method. The standard deviation of each value was estimated by the jack-knife method over loci as implemented in GENETIX version 4.02 (Belkhir *et al.,* 1999) and the linkage disequilibrium between loci was calculated using the LINKDIS procedure implemented in GENETIX (Belkhir *et al.,* 1999). Population structure was characterised using hierarchical F-statistics (theta) and G_{ST}-values as implemented in the GENETIX 4.02 software package (Belkhir *et al.,* 1999). Due to the subtle differentiation and the high number of rare alleles, we chose to estimate the fixation index ($F_{ST(RB)}$) following Robertson & Hill (1984) after correction by Raufaste & Bonhomme (2000). Significance of multi-locus F_{ST} was assessed with permutation tests (1000 replicates). Genetic diversity indices (H_E, H_O, MNA, MLH and P) were compared between individuals, river basins and *post-hoc* defined LOW-HIGH pollution groups (LOW = IMBI < 0.22 and HIGH = IMBI > 0.25, values of 0.22 < IMBI < 0.25 were removed to avoid overlap between both groups). Because of the absence of reproductively

isolated populations within each river basin (Maes & Volckaert, 2002; Tesch, 2003), the proposed division by pollution load is justified. In all cases significance levels were corrected for multiple comparisons using a sequential Bonferroni correction (Rice, 1989). Locus-by-locus heterozygosities (H_O), Allelic richness (AR) were compared using a pairwise t-test for dependant samples, while individual MLH values of both pollution groups were compared using an ANOVA. Bivariate regression analyses helped us to assess the relationship between condition and genetic estimators. Subsequent multiple regression analysis (Sokal & Rolf, 1997) was performed to test the overall contribution of MLH (allozymes and microsatellites) and condition (CI and HSI) on the level of bioaccumulation (IMBI). Analyses were performed in STATISTICA version 6.0 (StatSoft).

RESULTS

Heavy metal bioaccumulation - Comparisons of heavy metal pollution between river basins pointed to a strong heterogeneity in pollution load between sites (MANOVA, $F_{14,138} = 5.044$, $p < 0.0001$, $N = 78$, (see Appendix 1 and Table 1). The metals As and Se were not included in the statistical treatment because of the low number of analyses performed (1-5 individuals per river basin).

Table 1: Average heavy metal concentration per river basin of *Anguilla anguilla*. Multivariate and univariate ANOVAs for equal heavy metal bioaccumulation in eel tissue originating from the Yser, Meuse and Scheldt basin. Values for Hg, Cd, Pb, Ni, Cr, As and Se are expressed in $\mu g.kg^{-1}$. Values for Cu and Zn are expressed in $mg.kg^{-1}$. The highest values are listed in bold.

Basin	*N*	Hg	Cd	Pb	Cu	Zn	Ni	Cr	As	Se	All metals
Yser	25	150.32	2.448	41.68	0.518	23.88	46.52	295.68	135	329	W-value = 0.438
Meuse	20	**173.6**	**19.485**	37.6	0.493	**26.31**	**65.7**	**361.5**	**371.25**	663.5	F = 5.044
Scheldt	33	93.6	2.993	**52.78**	**0.643**	25.14	46.54	174.36	308.67	**1022.8**	df = 14
p-value		0.0006	0.0000	0.4600	0.5222	0.5152	0.1482	0.3818	/	/	0.0000

The Meuse basin exhibited the highest concentrations for six out of nine heavy metal measurements (when including As and Se), but only two out of seven heavy metal (Hg, Cd) concentrations differed significantly between river basins (ANOVA, Table 1). The Scheldt showed the lowest Hg concentration, while the Meuse had the highest level of Cd. The distribution of heavy metal concentrations was heterogeneous between sites within river basin (Appendix 1), as shown in figure 2a. The distribution of the IMBI values (based on seven

metals) ranged from 0.113 to 0.479 and showed a roughly bimodal pattern of lowly and highly polluted individuals (Figure 2b).

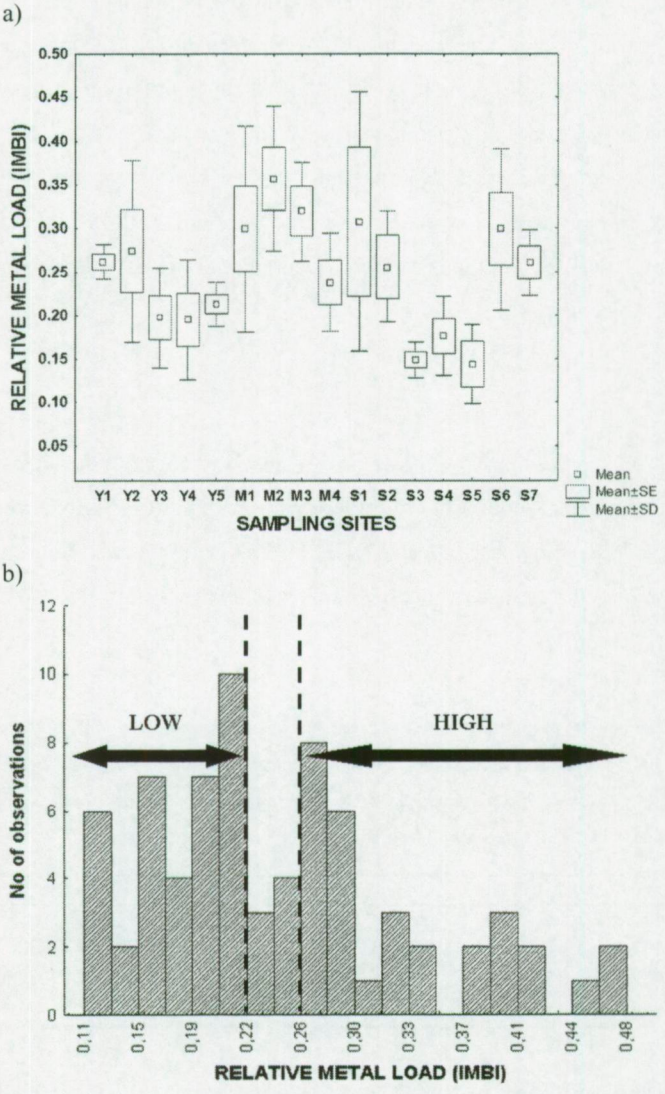


Figure 2: Level of heavy metal pollution (IMBI) in *Anguilla anguilla*. a) Per sampling site within river basin (upper panel); b) Histogram: the "HIGH" and "LOW" group are defined from the bimodal distribution of IMBI values (lower panel).

Later on this separation was used in the genetic analyses to define the "HIGH" and "LOW" pollution groups. An ANOVA of the IMBI values followed by a Tukey test indicated the Meuse basin as being significantly stronger polluted than the other two rivers ($F_{2,75} = 6.834$,

$p < 0.01$, N = 78, Figure 3). Considering the possible relationship between size and pollution load (length is only weakly correlated with age in yellow eels), we found no significant correlation between length and pollutant concentration for any heavy metal (data not shown).

Condition (CI) and Hepatosomatic (HSI) indices – The regression equation between length and weight was log (W) = 3.155 log (L) - 3.032 (r = 0.9746, n = 123, $p < 0.001$). The relative condition index (CI) was calculated as $1000(W/L^{3.155})$ and varied significantly among basins (ANOVA, $F_{2, 120} = 10.565$, $p < 0.001$), with the Meuse showing the lowest condition (Figure 3). HSI values varied from 0.917 to 2.639 among basins. The correlation between Weight and HSI was r = -0.27; $p < 0.01$. The relative hepato-somatic index, measured as the residuals of the former regression, differed significantly among basins (ANOVA, $F_{2, 120} = 5.897$, $p < 0.01$), pointing to the Meuse river as exhibiting the lowest values (Tukey test).

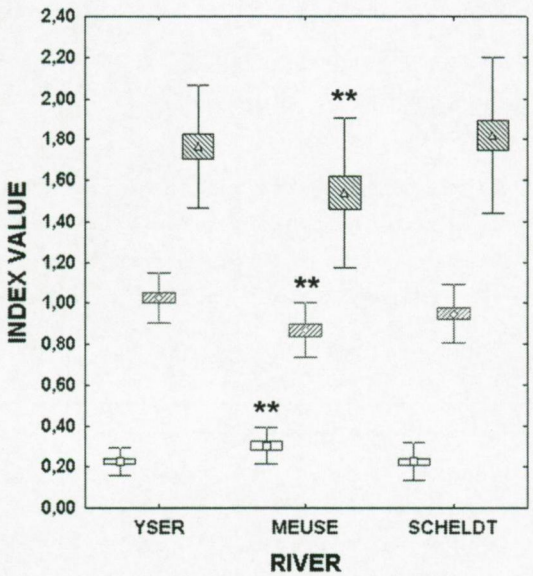


Figure 3: Boxplots representing relative condition index (CI ±S.D.), hepatosomatic index (HSI ± SD) and level of heavy metal pollution (IMBI ± SD) of *Anguilla anguilla* for each river basin. ** = $p < 0.01$

Finally, there was a significant negative correlation between heavy metal bioaccumulation (IMBI) and condition (r = -0.24; $p < 0.05$, Figure 4a) and a negative relationship between IMBI and HSI (r = -0.20; p = 0.09) (Figure 4b). No correlation was observed between CI and HSI (data not shown).

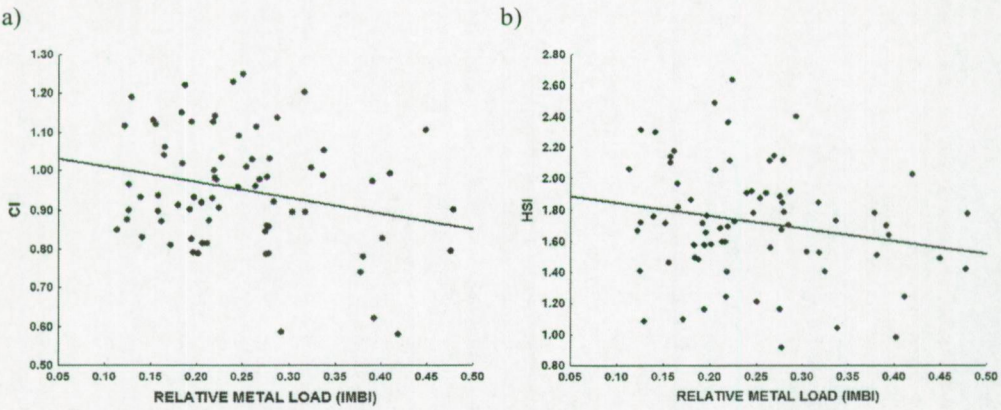


Figure 4: Correlation between heavy metal bioaccumulation (IMBI) of *A. anguilla* and a) condition index (CI) with R = - 0.24; p = 0.039; b) hepatosomatic index (HSI) with R = - 0.20; p = 0.09 for all individuals (N = 73).

Intra- and inter-basin genetic variability – A total of 12 enzymatic loci were scored. The total number of alleles per locus ranged from 1 to 6 and from 2.3 to 2.6 per sample over all loci. Observed (H_O) and expected (H_E) heterozygosities per sample ranged from 0.103 to 0.119 and from 0.122 to 0.129 respectively (Table 2; Appendix 2).

Table 2: Allozymatic and microsatellite genetic variability of *Anguilla anguilla* L. in the three river basins and in the LOW and HIGH pollution group. Expected (H_E) and observed (H_O) heterozygosity per sample/*post-hoc* group over all loci, level of polymorphism (P) and mean number of alleles (MNA) per sample/ *post-hoc* group over all loci. *N* : number of individuals; S.E.: standard error; $P_{(0.95)}$ or $P_{(0.99)}$: 95 % or 99% polymorphism criterion respectively. ** = $p < 0.01$.

Sample	N	H_E ±S.E.	H_O ±S.E.	$P_{(0.95)}$	$P_{(0.99)}$	MNA.	F_{IS}
Allozymes							
Yser	41	0.122 ± 0.154	0.111 ± 0.119	0.417	0.750	2.333	0.104
Meuse	41	0.129 ± 0.147	0.103 ± 0.107	0.500	0.750	2.583	0.212**
Scheldt	41	0.125 ± 0.165	0.119 ± 0.171	0.417	0.583	2.417	0.063
LOW	35	0.140 ± 0.159	0.131 ± 0.151	0.500	0.750	2.833	0.075
HIGH	32	0.109 ± 0.140	0.088 ± 0.094	0.417	0.667	2.333	0.212**
Microsatellites							
Yser	41	0.850 ± 0.073	0.792 ± 0.089	1.0	1.0	14.875	0.082**
Meuse	41	0.869 ± 0.068	0.822 ± 0.087	1.0	1.0	16.250	0.069**
Scheldt	41	0.851 ± 0.078	0.802 ± 0.054	1.0	1.0	14.375	0.072**
LOW	35	0.863 ± 0.073	0.803 ± 0.077	1.0	1.0	15.875	0.087**
HIGH	32	0.856 ± 0.080	0.817 ± 0.049	1.0	1.0	15.250	0.062**

An overall probability test of H&W equilibrium based on 1000 iterations was highly significant ($p < 0.001$), pointing to the non-random distribution of alleles within some samples. A global test based on 1000 iterations with H_I = heterozygote deficiency was only highly significant for the Meuse river (F_{IS}: 0.21; $p < 0.001$, Table 2), mainly due to loci *GPI-1**, *GPI-2**, *MDH-2** and *ADH-1**. A more detailed analysis of heterozygosities within river basin showed that the Meuse exhibited the highest expected heterozygosity ($H_E = 0.129$), the highest mean number of alleles (MNA = 2.58) and the highest level of polymorphism ($P_{(0.95)} = 0.50$). In contrast, this population exhibited the lowest observed heterozygosity ($H_O = 0.107$) (Table 2). No linkage disequilibrium was observed in the three populations.

The microsatellite loci revealed higher levels of variability than the allozymes as the total number of alleles per locus ranged from 12 (*AAN 05*) to 40 (*ANG 114*) and heterozygosity values (H_E) per locus ranged from 0.735 to 0.939 (Appendix 2). The mean number of alleles per locus by population varied between 14.4 (Scheldt) and 16.3 (Meuse). Observed and expected heterozygosity (H_O and H_E) per population were highly variable, ranging from 0.792 to 0.822 and from 0.850 to 0.869, respectively (Table 2). Exact tests assuming H_I = heterozygote deficiency, revealed significant departures from the null hypothesis of H&W equilibrium in all samples (Table 2). The deficits could be attributed to a particular locus, namely *AAN 02* which exhibited the strongest inbreeding coefficients ($F_{IS} = 0.22$, $p < 0.001$), most likely due to null alleles. Detailed analysis of population specific genetic variability defines the Meuse population as the most variable, with the highest mean number of alleles (MNA = 16.3), expected heterozygosity ($H_E = 0.869$) and observed heterozygosity ($H_O = 0.822$) (Table 2). No linkage disequilibrium was observed in the three populations.

Micro-scale genetic structure – Overall genetic differentiation was significant ($p < 0.05$), but the multi-locus unbiased differentiation estimators were very low for allozymes ($F_{ST(RB)} = 0.007$, $G_{ST} = 0.001$) and for microsatellites ($F_{ST(RB)} = 0.018$, $G_{ST} = 0.003$). Pairwise genetic differentiation shows discrepancies between both markers. The microsatellite genotypes of the Meuse basin are most distinct from the Yser ($F_{ST(RB)} = 0.025$, $p < 0.01$), while the allozyme genotypes differentiate Yser and Scheldt the most ($F_{ST(RB)} = 0.017$, $p < 0.05$).

Genetic composition of the "HIGH" and "LOW" pollution group – The bimodal distribution of the IMBI values allowed us to define two groups ranked by their magnitude of relative metal load (Figure 2b). A total of 67 individuals were ultimately selected, with 35 and 32

individuals in "HIGH" and "LOW" polluted condition respectively. To exclude redundancy, we tested for the independence between basin and HIGH-LOW pollution groups (Chi-square = 7.33; df= 5; $p > 0.05$). The "HIGH" pollution group clearly exhibited a lower allozymatic genetic variability (H_E, H_O, level of polymorphism and MNA) than the "LOW" pollution group (Table 2, Appendix 2). The proportional difference between both groups ($H_{LOW} - H_{HIGH} / H_{LOW}$) amounted to 21.5 % H_E and 34.6 % H_O between both pollution groups. Locus-by-locus heterozygosity (H_O) and allelic richness (AR) analysis points to a significantly lower AR (t-test, $p = 0.01$, Figure 5a) and lower H_O (t-test, $p = 0.03$, Figure 5b) for polluted individuals.

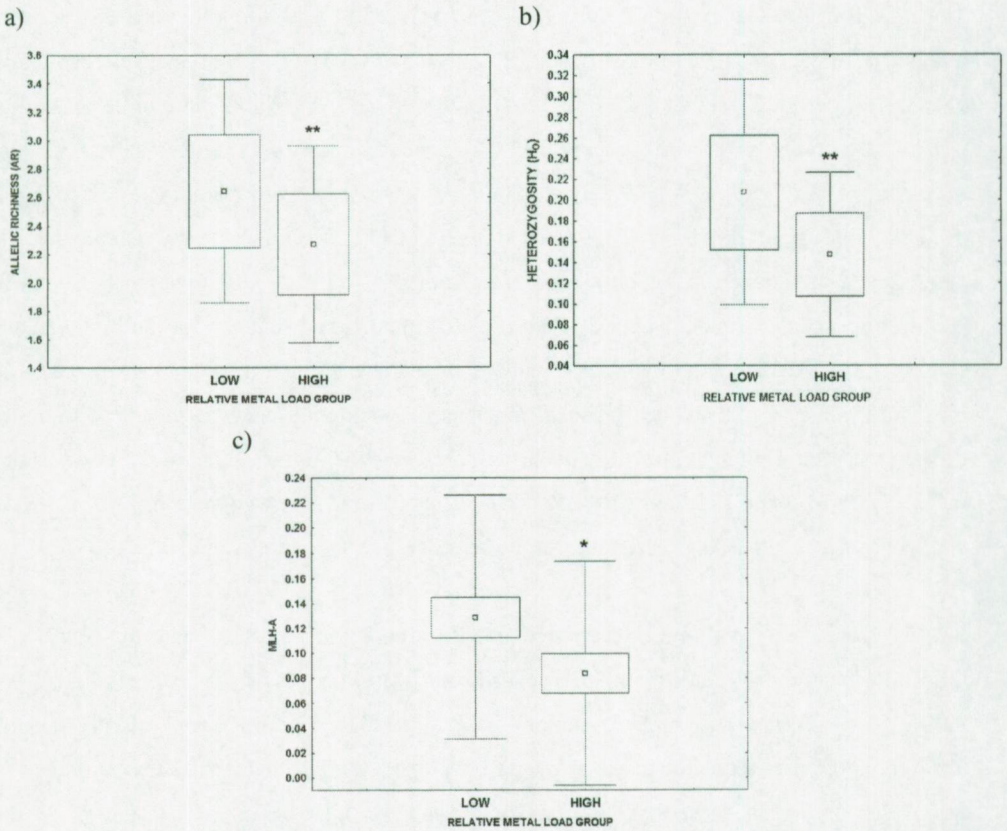


Figure 5: Allozymatic genetic variability of *A. anguilla*. Boxplots representing mean (± SE) a) allelic richness (AR, t-test; $p = 0.01$), b) observed heterozygosity (H_O; t-test; $p = 0.03$) locus-by- locus and c) Multi-locus Heterozygosity (MLH; ANOVA, $p = 0.05$) comparisons between "HIGH" and "LOW" heavy metal pollution groups. * = p = 0.05, ** = $p < 0.05$

Finally, the number of multi-locus genotypes (28 vs. 22) was higher in the "LOW" pollution group. In contrast, microsatellite variability (multi - and locus-by-locus analyses) showed no appreciable difference in expected or observed heterozygosity, allelic richness or number of alleles between both groups (data not shown). When individual MLH were compared for allozymes and microsatellites, we observed marginally significant lower allozyme MLH values for HIGH polluted individuals (ANOVA, $F_{1, 65} = 3.898$, $p = 0.05$), while for microsatellites no differences could be detected ($p > 0.05$) (Figure 5c).

MLH, condition and heavy metal load regression - Regression analysis between MLH (allozymes and microsatellites) and condition indices (CI and HSI) yielded a negative trend, but no significant correlation (N = 123, data not shown). Regression between individual allozyme MLH and IMBI values yielded a significant negative correlation ($R = -0.28$; $p = 0.016$), while microsatellite MLH was not correlated with IMBI ($R = 0.07$, $p > 0.05$) (Figure 6).

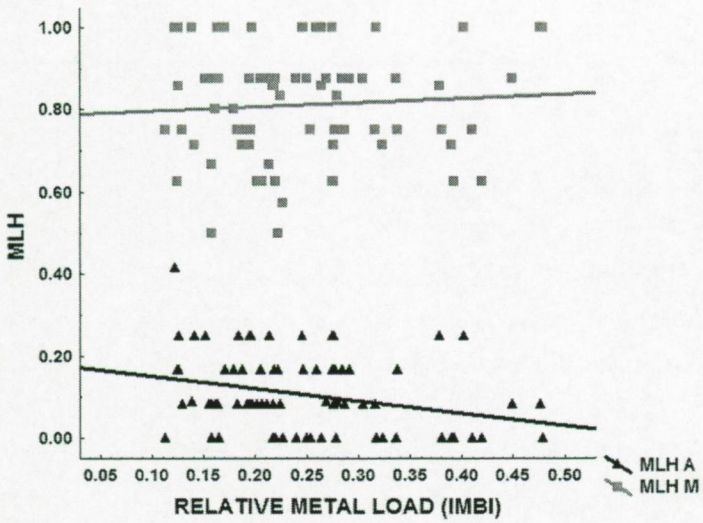


Figure 6: Correlation between relative heavy metal bioaccumulation (IMBI) and multi-locus heterozygosity (MLH) in *A. anguilla* for allozymes (MLH A) with R = -0.28; p = 0.016 and microsatellites (MLH M) with R = 0.07; p = 0.56.

Similarly, when performing a regression analysis using the mean IMBI and MLH values per sampling site (N = 16), a marginally significant negative correlation (R = -0.48, p = 0.05) is observed (data not shown), indicating that similar results can be obtained if

comparing individual sampling sites. Multiple regression analysis of IMBI as dependent variable versus MLH allozymes, MLH microsatellites, CI and HSI resulted in a better model to explain variation in bioaccumulation ($F_{4, 68} = 5.776$, $p < 0.001$; adjusted $R^2 = 0.21$) than each variable alone (see Table 3, Figure 6). All variables, except the MLH of microsatellites, were correlated with IMBI values. MLH of allozymes contributed the most (β-weights) to the variation in IMBI, followed by CI, HSI and MLH of microsatellites (Table 3).

Table 3: Multiple regression analysis relating individual heavy metal bioaccumulation (IMBI) to condition (CI and HSI) and multi-locus heterozygosity (allozymes; MLH-A and microsatellites; MLH-M). The relative contribution of each variable is reported as beta-weight (standardized regression coefficient). Standard errors are given in parentheses.

	Parameter (s.e.)	df	t	p-value	Beta weights (s.e.)
Intercept	0.535 (0.100)	1	5.352	0.000	/
CI	-0.210 (0.066)	1	-3.185	0.002	-0.343 (0.108)
HSI	-0.070 (0.026)	1	-2.653	0.010	-0.285 (0.107)
MLH- A	-0.350 (0.100)	1	-3.486	0.001	-0.376 (0.108)
MLH- M	0.086 (0.071)	1	1.209	0.231	0.129 (0.107)
Error	/	68	/	/	/

DISCUSSION

Although the impact of pollution on genetic variability has been assessed previously (Bickham *et al.*, 2000; Belfiore & Anderson, 2001 for a review), this study is novel in several aspects. First, our study focused on the level of bioaccumulation in a species extremely prone to pollution due to its high fat content, reflecting the actual pollution stress in the organism (Collings *et al.*, 1996). Its catadromous life history enables the detection of local pollutant influences on somatic and genetic characteristics, as juveniles enter rivers with much less pollution load or differential genetic background than locally reproducing and genetically distinct freshwater species. Their level of bioaccumulation after a few years spent in the rivers can be considered as indicative of their fitness, because strongly polluted eels detoxify less efficiently, have a lower condition and might be less successful spawners (Feunteun, 2002). Secondly, it has been suggested that several genetic markers should be used to discriminate between the influence of selection and other factors that might be marker specific (Belfiore & Anderson, 2001). In this study we compared patterns from strictly neutral genetic markers (microsatellite DNA) with enzymatic markers (allozymes), for which the assumption of selective neutrality has often been challenged (Eanes, 1999). The significance of assessing

biometric (weight, condition, growth) responses has also been underlined as a measure of pollutant impact on the organism (Van Straalen & Timmermans, 2002). Finally, the study of highly vagile organisms with a catadromous life-history like eel remains underrepresented, due to the difficulty of defining biologically relevant populations.

Earlier studies used reproductively isolated populations, enabling straightforward population comparisons in the light of the "genetic erosion" hypothesis (Van Straalen & Timmermans, 2002). Here, we explain this issue in two ways, namely (1) by assessing the impact of pollutants on genetic variability ("Genetic Erosion" hypothesis) and (2) by considering individual genetic variability as an advantage to cope with pollution ("Heterosis" or "overdominance" hypothesis). Nevertheless, due to the catadromous life-history of eel and its failure to breed in captivity, no strong conclusions about evolutionary consequences can be drawn from our observations.

Spatial heterogeneity in pollution and condition

Although the European eel is a highly vagile fish species (Tesch, 2003), the feeding stage inhabiting the freshwater environment is remarkably sedentary and pollutants are expected to have a local influence. Our results confirm this knowledge; the accumulation of heavy metals is strongly heterogeneous between and within basins (Table 1, Figure 2). We found significant differences between individuals originating from other river basins, pointing to locally highly and less polluted sites. Despite intra-riverine variability, the Meuse basin was the strongest polluted river (Table 1), in line with current perception (Maeckelberghe, 2003; Cellule Etat de l'Environnement Wallon, 2003). The bioaccumulation of heavy metals, defined as a relative index (IMBI), confirmed single metal predictions, namely pointing to the Meuse eels as significantly stronger polluted than the eels of other basins. Earlier studies on European eel have confirmed the heterogeneous distribution of pollutants in rivers and lakes (Linde *et al.*, 1996; Belpaire *et al.*, 2002; Goemans *et al.*, 2003). The individual level of bioaccumulation might provide both an estimate of the environmental quality of the sediment (eels are benthic feeders) and a measure of health condition (fitness) of the organism (Bervoets & Blust, 2003). As no correlation was found between eel length and pollutants, the capacity of detoxification of individuals seemed unrelated to their size.

Life-history traits, such as condition, growth and fecundity, reflect the environmental quality and the organism's historic experience (Meffe, 1991; Ridley, 1996). We expected an impact of the level of bioaccumulation on the condition of European eel, due to the excess

energy required for detoxification. We clearly showed a relationship between an increased heavy metal content and a lower condition in eel. The Meuse population exhibited a significantly lower condition than the other two river basins. Regression analysis revealed a strong negative correlation between individual bioaccumulation and condition indices (Figure 4a and b), which confirms the literature. For example, in the Sydney rock oyster (*Saccostrea commercialis*), bioaccumulation strongly correlated with condition (Avery *et al.*, 1996). Hence, we have strong indications that the bioaccumulation of heavy metals is a predictor of the condition in European eel and that pollution might significantly affect individual fitness. Due to the mobilisation of fat reserves during the spawning migration, it is expected that highly polluted individuals will have a lower reproductive success during spawning (van Ginneken & van den Thillart, 2001; Robinet & Feunteun, 2002)

Bioaccumulation vs. intra river and post-hoc genetic variability

Because of the absence of reproductively isolated groups in Belgium and Europe (Wirth & Bernatchez, 2001; Maes & Volckaert, 2002), it remains difficult to sustain the concept of "populations" in a river basin. Analyses performed at the population level are mostly testing for Hardy-Weinberg equilibrium, which can also be interpreted as randomness in genotypic distribution within rivers instead of random mating amongst individuals. Our proposal to analyse on the one hand natural populations (river basin) and on the other hand phenotypic traits (pollution charge), aims first at analysing the influence of a geographically divergent pollution level (local pattern of genetic variability) and subsequently mainly at comparing the genetic variability based on pollutant concentration in "general", where an individual's heterozygosity determines its response to pollutants. The level of bioaccumulation does not necessarily reflect the environmental pollution but also the individual capacity for detoxification. Hence, we argue that individual bioaccumulation is also determined by the genetic make-up.

Considering genetic variability within a river basin, we showed that the Meuse, despite exhibiting the highest expected variability, was in strong H&W disequilibrium, pointing to a non-random distribution of genotypes and possibly differential selection. Such results may have several causes, like population substructure, null alleles, inbreeding and selection (Hartl & Clarck, 1997). Because of the absence of reproductively active populations and the lack of similar results on microsatellites (excluding locus AAN 02), selection seems the most plausible explanation for the genotypic shift. The most strongly polluted population was the

least heterozygous (H_O) at allozymes, possibly attributing weaker detoxification ability to more homozygous individuals and/or shifts towards certain homozygote classes.

Due to the heterogeneous distribution of metals and the absence of "biological" populations in rivers, we ranked individuals in *post-hoc* groups according to their level of bioaccumulation. The pattern exhibited here was much more unambiguous, namely a lower overall genetic allozymatic variability in strongly polluted individuals and again a strong H&W disequilibrium. A similar decrease in genetic variability has been demonstrated in various other freshwater, marine and terrestrial organisms under natural and laboratory conditions (Hvilsom, 1983; Fevolden & Garner, 1986; Klerks & Weis, 1987; Posthuma & Van Straalen, 1993). Changes in diversity were mostly attributed to the selective advantage of certain genotypes or a reduction in population size (N_e). Remarkably, in most studies either only a few enzymatic loci were screened or an impact was observed at few loci (Chagnon & Guttman, 1989; Gillespie & Guttman, 1989; Patarnello & Battaglia, 1992; Newman & Jagoe, 1998). In the present study, we observed a multi-locus response on pollution, namely at seven out of nine enzymatic loci. Only locus *GPI-1** remained constant, while *MPI-1** even exhibited a higher variability in strongly polluted individuals, possibly pointing to a heavy metal tolerant allele. We observed a lower number of genotypes in the highly polluted group, which fits the expectations (Ben-Shlomo & Nevo, 1988; Chagnon & Guttman, 1989; Diamond *et al.*, 1991), and suggests differential mortality or genotype shifts. Interestingly, 74 % of Meuse individuals belong to the HIGH pollution group compared to 40 % and 35 % for the Yser and Scheldt respectively. This confirms the lower observed variability (H_O) in the Meuse, while the remaining low polluted individuals from this river may have raised the MNA and hence the expected heterozygosity by carrying rare alleles. The genetic variability at strictly neutral markers did not show any pollution related differences, despite the high number of alleles and the higher resolution expected from this marker (Hedrick, 1999). Nevertheless, other studies using similar markers have found a strong correlation between a decrease in neutral genetic variation and the level of pollution in natural aquatic (Nadig *et al.*, 1998; Krane *et al.*, 1999; Ma *et al.*, 2000; Matson *et al.*, 2000) and terrestrial (Theodokaris *et al.*, 2001) habitats. This result was somewhat expected as the only selection possibly influencing the genetic pattern of eel is direct selection on metabolically important enzymes, as microsatellites evolve strictly neutrally, mainly enabling the detection of post-reproductive selection (Bickham *et al.*, 2000; Belfiore & Anderson, 2001).

Bioaccumulation vs. individual genetic variability

A comparison between individual-based pollution characteristics and population summary statistics (H_E, P, MNA, H&W equilibrium) holds several difficulties for the interpretation, due to the assumption of "population" in genetic estimators. Therefore, we chose to analyse the relationships between all variables using individual based regression analyses. A negative correlation was observed between IMBI and condition indices, as well as between IMBI and allozymatic MLH. This suggests that strongly polluted individuals need more energy for detoxification and are on average in a worse condition, while more heterozygous individuals may accumulate less (Van Straalen & Kammenga, 1998). Increased fitness with heterozygosity has been empirically demonstrated in a large number of plants and animal species (see David, 1998 for a review), as heterozygotes are better buffered against environmental fluctuations, are superior due to their multimeric enzymes (Nevo *et al.*, 1986) and have a lower energetic demand, favouring such individuals in strongly polluted conditions (heterosis). Due to the multi-locus response or cause of the correlation, an overall metabolic gain in efficiency may be proposed as cause for the correlation (Eanes, 1999); most allozymes studied belong to the glycolysis or citric acid cycle. No Heterozygosity Fitness Correlation (HFC) was found at microsatellites, results concordant with recent findings in farmed eel where growth rate was correlated to allozymatic but not to microsatellite MLH (Pujolar *et al.*, 2005). The relative importance of condition and genetic variability to explain differences in heavy metal bioaccumulation as assessed by multiple regression analysis, pointed to allozymatic MLH, followed by the condition index as the main factors influencing bioaccumulation. Hence, an individual's enzymatic heterozygosity (and not necessarily its genome-wide heterozygosity) seems to play an important role in the potential to counteract pollutant bioaccumulation.

Conclusion

We clearly showed a strong correlation between the level of bioaccumulation and a reduced condition within resident eel populations. We also found an obvious link between pollution and a lower allozymatic genetic variability at the individual level and in two *post-hoc* defined groups of different pollution levels. Microsatellite variability did not reflect any pollution or condition related trend, and no individual HFC pattern. We hypothesize that enzymatic genetic variability (MLH) is a key issue to explain differences in the bioaccumulation of toxicants (or detoxification success), in other words to retain fitness. Hence, direct

overdominance seems the most likely explanation for the observed pattern in eel and thus not associative overdominance or genetic erosion (only detectable after reproduction). Complementary sampling and experimental studies should increase our confidence about the strength of ecological consequences in catadromous organisms, as well as about the heterosis effect (HFC) detected in this study. Conditional is the optimisation of artificial breeding before evolutionary inferences can be made experimentally. Our results also underline the complexity of evolutionary toxicology research in diadromous species, which switch between habitats. The knowledge of the genetic make-up is crucial to infer evolutionary consequences of pollutants in such species, which is only possible when assessing the interaction between ecology and genetics.

ACKNOWLEDGMENTS

This research was funded by The Ministry of the Flemish Community (AMINAL). Many thanks go to K. Peirsman for organizing the fishing and processing of the samples. We thank J.P. Nash, J.K.J. Van Houdt and two anonymous referees who helped improve the quality of this manuscript. G.E. Maes received a PhD scholarship from the IWT (Institute for the Promotion of Innovation by Science and Technology in Flanders). J.A.M. Raeymaekers received a PhD fellowship of the Fund for Scientific Research (F.W.O.-Vlaanderen). C. Pampoulie received a post-doctoral fellowship from FWO-Vlaanderen and the KULeuven.

Appendix 1: Average heavy metal concentration per kg of wet *Anguilla anguilla* tissue per sampling site: N = number of individuals analysed for heavy metals. Values for As and Se correspond to one individual at the respective sampling site. Values for Hg, Cd, Pb, Ni, Cr, As and Se are expressed in $\mu g.kg^{-1}$. Values for Cu and Zn are expressed in $mg.kg^{-1}$.

River	Sampling site	N	**Hg**	**Cd**	**Pb**	**Cu**	**Zn**	**Ni**	**Cr**	**As**	**Se**
YSER	Y1	5	245.20	2.60	49.80	0.37	25.76	46.00	171.00	/	/
YSER	Y2	5	59.60	1.78	75.40	1.20	19.20	77.20	632.40	135.00	329.00
YSER	Y3	5	139.40	2.18	24.80	0.39	23.78	37.80	285.60	/	/
YSER	Y4	5	194.60	2.58	20.00	0.37	27.36	17.20	146.60	/	/
YSER	Y5	5	112.80	3.10	38.40	0.33	23.30	54.40	242.80	/	/
MEUSE	M1	6	152.50	23.15	10.33	0.68	27.22	55.67	823.67	263.00	1081.00
MEUSE	M2	5	215.40	22.72	39.20	0.53	30.24	94.00	138.40	168.00	743.00
MEUSE	M3	4	175.20	22.58	28.00	0.33	23.92	82.20	157.60	733.00	488.00
MEUSE	M4	5	144.20	5.16	83.60	0.36	23.84	27.00	197.20	321.00	342.00
SCHELDT	S1	3	70.33	1.67	85.67	0.83	32.50	92.00	183.67	243.00	667.00
SCHELDT	S2	3	142.00	1.50	95.33	0.70	23.83	37.33	135.33	257.00	913.00
SCHELDT	S3	5	91.20	3.86	5.00	0.58	19.74	27.00	139.00	229.00	1064.00
SCHELDT	S4	5	66.00	1.50	55.60	0.83	24.46	5.00	200.20	/	/
SCHELDT	S5	3	99.67	1.50	15.67	0.50	17.00	25.00	187.33	254.00	1166.00
SCHELDT	S6	5	66.20	6.98	85.60	0.57	28.90	90.00	174.60	/	/
SCHELDT	S7	4	127.75	1.50	73.75	0.67	24.75	61.25	181.25	704.00	1556.00

CHAPTER VII:

Growth rate correlates to individual heterozygosity in European eel, *Anguilla anguilla* L.

Pujolar J.M., Maes G.E., Vancoillie C. and Volckaert F.A.M.

SUMMARY

Heterozygosity-fitness correlations (HFCs) have been reported in populations of many species. We provide evidence for a positive correlation between genetic variability and growth rate at 12 allozyme loci in a catadromous marine fish species, the European eel (*Anguilla anguill*a L.). More heterozygous individuals show a significantly higher length and weight increase and an above average condition index in comparison with more homozygous individuals. To a lesser extent, 6 microsatellite loci show a similar pattern, with positive but not significant correlations between heterozygosity and growth rate. The HFCs observed could be explained either by an effect of direct allozyme overdominance or associative overdominance. Selection affecting some of the allozyme loci would explain the greater strength of the HFCs found at allozymes in comparison with microsatellites, and the lack of correlation between MLH at allozymes and MLH at microsatellites. Associative overdominance (where allozyme loci are merely acting as neutral markers of closely linked fitness loci) might provide an explanation for the HFCs if we consider that allozyme loci have a higher chance than microsatellites to be in linkage disequilibrium with fitness loci.

Keywords: allozymes, associative overdominance, direct overdominance, heterozygosity, growth rate, microsatellites, selection

This chapter is published in: Evolution 59(1): 189-199.

INTRODUCTION

Heterozygosity-fitness correlations (HFCs), the correlation between heterozygosity observed at marker loci and fitness-related traits such as growth, survival, fecundity or developmental stability, have been under study for decades in populations of many species. Positive HFCs have been reported in organisms as diverse as plants (Schaal & Levin, 1976; Ledig *et al.*, 1983), marine bivalves (Zouros *et al.*, 1980; Koehn & Gaffney, 1984), crustaceans (Bierne *et al.* 2000), amphibians (Pierce & Mitton, 1982), salmonids (Leary *et al.*, 1984; Danzmann *et al.*, 1987; Thelen & Allendorf 2001) and mammals (Slate & Pemberton 2002; Hildner *et al.* 2003). Null results are likely to be under-represented in literature (Hansson & Westerberg 2002). The correlation between genetic variability and fitness components as reflected by molecular marker heterozygosity in natural populations usually accounts for a small percentage (1-5%) of the observed phenotypic variance (David, 1998).

The first HFCs were observed in studies using allozyme markers, which led to the hypothesis of direct overdominance, where the correlation is due to a direct heterozygous genotype advantage at allozyme loci compared to the corresponding homozygous genotypes. It has been proposed that heterozygotes at allozyme loci might have an intrinsically higher fitness than homozygotes, which would be related to lower energy consumption and greater metabolic efficiency (Mitton, 1993).

The observation of positive HFCs with putative neutral DNA markers proves that at least some correlations are not due to the direct effect of the marker genes but to the genetic association between the neutral markers and fitness genes, resulting in associative overdominance (David, 1998). When marker loci are not directly responsible, the observed correlation can be either due to the effect of linkage disequilibrium restricted to a narrow chromosomal section affecting closely-linked fitness loci ("local effect") or due to partial inbreeding caused by the non-random association of diploid genotypes in zygotes ("general effect") (David *et al.*, 1995).

Few studies correlating heterozygosity and growth have been carried out in fish, mainly due to the difficulty in rearing experiments, especially for marine species. In salmonids, Danzmann *et al.* (1987) found a positive correlation between allozyme heterozygosity and growth (length or weight up to 6 months) in cultured rainbow trout (*Oncorhynchus mykiss*), suggesting that heterozygotes have enhanced growth rates compared to more homozygous individuals. Nevertheless, salmonids seem to produce different HFCs at different life stages, and negative correlations were found between (1) allozyme variation and length at one year,

and (2) length and weight at maturation (Ferguson, 1990, 1992). Pogson & Fevolden (1998) examined the relationships between growth and the degree of individual heterozygosity at ten nuclear RFLP loci in two natural populations of Atlantic cod (*Gadus morhua*), using a rough measure of growth (size at age). A significant positive correlation was found in one population, supporting the hypothesis that neutral DNA markers can detect HFCs.

The species of interest in this study is the European eel (*Anguilla anguilla* L.; Anguillidae; Teleostei), a catadromous fish species with a particularly complex life cycle, which moves between marine (spawning, larval phase and maturation) and freshwater (feeding, growth and maturation) environments. After spawning in the Sargasso Sea, larvae (leptocephali) migrate with the Gulf Stream and North Atlantic Drift Current to the shores of Europe and North Africa, where they metamorphose into glass eels upon reaching the continental shelf. Glass eels move into freshwater systems (rivers, lagoons or lakes), where they feed for up to 25 years (on average, 7-8 years for males and 11 years for females) before metamorphosing into silver eels and migrating back to the Sargasso Sea, where they spawn once and die (Tesch 2003).

Recruitment abundance of European eel has declined dramatically in recent decades, jeopardizing the future of the species (Dekker 2003). A better understanding of crucial aspects of its biology, including genetic diversity, may promote effective measures to protect the species. In the case of European eel, no previous studies have been carried out to correlate individual growth and genetic variability. Despite progress in artificial propagation, completion of the European eel lifecycle in captivity is not possible yet (Tesch 2003). Alternatively, fattening farms dedicated to the growth of stocked glass eel (varying from postlarvae having completed the leptocephalus metamorphosis to the full pigmentation stage), are readily available in many coastal regions of southern and western Europe. There is a long history of stocking and extensive rearing of eels in ponds, offering an adequate setting for growth experiments (Tesch 2003). When growth is tested under artificial conditions, it must be taken into account that environmental stress might enhance HFCs (Danzmann *et al.*, 1988) and that HFCs seem to decrease with age (David, 1998).

The aim of this study is to identify the existence of HFCs in European eel in order to evaluate genetic variability and selection in cultured eel populations and to assess the contribution of genetic and environmental factors in the growth of the species. At the same time, such experiments provide a model for natural populations of European eel and for other fish species. The association between heterozygosity and two phenotypic measures of growth (length and weight increase) was tested in two groups of farmed European eel individuals

using thirteen allozyme and six microsatellite markers. In case of direct overdominance, HFCs should not be expressed at putative neutral markers (microsatellites) but only at markers affected by natural selection (allozymes). Under the hypothesis of associative overdominance, an apparent advantage observed at heterozygous individuals would be independent of the genetic marker used. Since linkage is not limited to allozymes, a similar HFC would be expected using either allozyme or microsatellite markers. Both hypotheses are not mutually exclusive since natural selection might not be acting equally across all allozyme loci. Besides, despite the usual interpretation that microsatellites evolve neutrally, recent studies have documented the functional role of microsatellites in gene regulation, chromatin organization, cell cycle and DNA metabolic processes (Li *et al.*, 2002).

MATERIAL AND METHODS

Glass eel individuals collected in the mouth of the river Adour (South-western France) were transferred and raised in a closed recirculation system at the eel farm of Royaal BV (The Netherlands). Two batches of glass eels, collected in December 2001 (Batch 1; ROY101) and February 2002 (Batch 2; ROY 201), respectively, were monitored for one year. A sub-sample of 100 individuals was collected from each batch at the start of the experiment and after one year in the tanks (ROY102 and ROY202) for genetic analyses (Table 1). Once measured, individuals were split in two, with the tail kept in ethanol for microsatellite analysis and the rest of the body kept frozen at –80°C for allozyme analysis.

Table 1. *Anguilla anguilla.* Summary of genetic samples of farmed glass eels including sampling date, number of individuals analysed (N), mean length (L) and mean weight (W). Standard deviation in parentheses.

Sample	Batch	Sampling date	N	Mean L (mm) ± S.D.	Mean W (g) ± S.D.
ROY101	1	December 2001	100	71.69 (3.80)	0.41 (0.07)
ROY102	1	December 2002	100	233.04 (69.80)	29.16 (31.95)
ROY201	2	February 2002	100	70.36 (4.47)	0.29 (0.07)
ROY202	2	February 2003	100	241.34 (87.74)	43.37 (44.80)
ROY2F	2	February 2003	50	612.9 (41.1)	602.2 (117.5)

Additionally, genetic analysis also included a sub-sample of 50 individuals from batch 2 (ROY2F), which were regarded as "fast-growth individuals" due to their exceptional growth rate after one year in the tanks in the same conditions as the rest of the individuals (mean length: 612.9 ± 41.1 mm/ mean weight: 602.2 ± 117.5 g).

Allozyme electrophoresis – A total of 450 individuals (Table 1) were analysed for protein variation using Cellulose Acetate Gel Electrophoresis (CAGE, Harris and Hopkinson, 1976; Richardson *et al.*, 1986). Tissue extraction, electrophoresis and procedures for visualising proteins, and buffer systems used (Tris Glycine (TG) and Tris Malate (TM)) are described in Maes and Volckaert (2002). Nine enzymatic systems were examined: aspartate aminotransferase (*AAT-1**, *AAT-2**, *AAT-3**, EC 2.6.1.1, TM), alcohol dehydrogenase (*ADH**, EC 1.1.1.1, TG), glucose-6-phosphate isomerase (*GPI-1**, *GPI-2**, EC 5.3.1.9, TG), L-Iditol dehydrogenase (*IDDH**, EC 1.1.1.14, TG), isocitrate dehydrogenase (*IDHP**, EC 1.1.1.42, TM), malate dehydrogenase (*MDH-2**, EC 1.1.1.37, TM), mannose-6-phosphate isomerase (*MPI**, EC 5.3.1.8, TG), phosphogluconate dehydrogenase (*PGDH**, EC 1.1.1.44, TM) and phosphoglucomutase (*PGM**, EC 5.4.2.2, TG). Genetic nomenclature followed the suggestions of Shaklee *et al.* (1990). Allele assignment was carried out comparing the relative distance with the most common allele (**100*).

Microsatellite analysis – DNA was extracted in a sub-sample of 60 out of the 100 individuals from each batch at the start of the experiment and after one year in the tanks, and 50 fast-growth individuals. Minute sections of tissue from ethanol preserved glass eels were digested in a lysis buffer containing 100 µl TE Buffer, 7 µl 1M DTT (Dithiothreitol) solution pH 5.2 (diluted in 0.08M NaAc) and 5 µl Proteinase K solution (10 $mg.ml^{-1}$) for at least 4 h at 56°C. After incubation at 96°C for 10 min, samples were centrifuged at 13,000 rpm for 11 min; the supernatant was stored at –20°C for further analysis.

Genotypes were examined at 6 dinucleotide repeat microsatellite loci: AAN 01, AAN 03, AAN 05 (Daemen et al., 2001); ARO 063, ARO 095 and ANG 151 (Wirth & Bernatchez 2001). Loci were amplified in two separate multiplexes. Multiplex PCR reactions consisted of 1 X PCR buffer (supplied with polymerase), 1.5 mM $MgCl_2$, 80 µM dNTP, 0.4 µM (on average) fluorochrome labelled forward and non-labelled reverse primer, 0.5 U Goldstar *Taq* polymerase (Eurogentec) and 1 µl DNA template. DdH_2O was added up to 25 µl. PCR cycling conditions were as follows: 5 min at 94°C, 24 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, and final elongation for 8 min at 72°C. PCR products were visualized on an automated sequencer (LICOR 4200), using a molecular ladder (Westburg) in order to quantify allele sizes. Fragment data were analysed using Gene ImagIR ver 4.03 (Scanalytics Inc).

Data analysis – All individuals were measured for standard length (L) and body weight (W). Length and weight increase are calculated as the difference between individual length and

weight after one year in the tanks and mean length and weight after arriving in the facilities. Ricker's (1975) condition factor ($CI= 1000(W/L^b)$) was calculated for each individual, where L is standard length in mm, W is body weight in mg and b is the slope from the log length-log weight regression for all samples. The condition factor is based on the analysis of length-weight data, assuming that heavier individuals at a given length are in a better condition. Additionally, the relative condition factor (K) has been calculated as described in Le Cren (1951). $K= W/\hat{W}$ compares the observed (W) and expected ($\hat{W}$) weight of each individual, where expected weight is obtained using the length-weight regression ($W=aL^b$) of each individual. Differences in morphometric measures among groups were tested by an analysis of variance (ANOVA).

Within sample genetic variation was assessed by observed heterozygosity per locus (H_o), level of polymorphism using the 95% criterion ($P_{0.05}$) and mean and total number of alleles using GENETIX version 4.02 (Belkhir *et al.*, 1999). Values among groups were compared by an analysis of variance (ANOVA). Deviations from Hardy-Weinberg equilibrium, genotypic associations for all possible pairs of loci in each sample, and differences in allele frequencies among samples were tested using GENEPOP version 3.1d (Raymond & Rousset, 1995). Significance levels for multiple simultaneous comparisons were adjusted using the sequential Bonferroni technique (Rice, 1989). Patterns of variation among samples were assessed by hierarchical gene diversity analysis as implemented in GENETIX version 4.02 (Belkhir *et al.*, 1999).

Individual multi-locus heterozygosity (MLH) was calculated as the proportion of loci that are heterozygous (corrected for non-scored loci). MLH among groups was compared using an ANOVA. In the case of microsatellite loci, the squared difference in repeat units between two alleles in an individual at a given locus (d^2) averaged over all loci (mean d^2) was calculated following Coulson *et al.* (1998). Mean d^2 is an alternative method to MLH in order to infer fitness differences between individuals; in addition to taking into account whether individuals are homozygotes or heterozygotes, it also considers the differences in allele size in heterozygous individuals. Coltman *et al.* (1998) suggested that mean d^2 provides a better measure of individual genetic variability than MLH for microsatellite data. Outbreeding mean d^2 (calculated in the same way as mean d^2 but excluding homozygous loci in each individual) and standardized mean $d^2_{var\ (d2)}$ (the average d^2 at each locus scaled by the variance of d^2 at that locus) were calculated for each individual as described by Hedrick *et al.* (2001).

All data were checked for normality and a logarithmic transformation was conducted if necessary before parametric statistical analysis. Regression analysis (Pearson's correlation)

was performed between individual heterozygosity (MLH and all calculations of d^2) values and growth estimators (length increase, weight increase, condition index and relative condition index) in order to test for possible HFCs. When fast-growth individuals were included in the analysis, Spearman's correlation was used since variables did not approximate a normal distribution following logarithmic transformation. All analyses were performed in STATISTICA version 6.0 (Statsoft). Significance for all statistical tests was taken as 0.05.

RESULTS

A comparison between the newly arrived batches 1 and 2 showed no significant differences in length and weight (Table 1). After one year at the farm, a substantial range of sizes was observed in both batches. In batch 1, average length was 233.04 ± 69.80 mm, with a maximum length of 444 mm and a minimum length of 135 mm. Differences were not significant in comparison with batch 2, with an average length of 241.34 ± 87.74 mm ranging from 110 - 433 mm. Larger differences were observed in the weight distribution, with individuals from batch 2 (mean weight: 43.37 ± 44.80 g; maximum weight: 183.2 g) being heavier than individuals from batch 1 (mean weight: 29.16 ± 31.95 g; maximum weight: 136.2 g), although differences were not significant. Individuals from batch 1 showed an average increase of 167.58 ± 66.83 mm and 29.29 ± 31.48 g, while individuals from batch 2 showed an average increase of 176.18 ± 84.97 mm and 44.45 ± 44.62 g. Increases of both batches were not significantly different.

In order to correlate genetic variability with growth, the samples from batch 1 and 2 which had spent one year at the farm (ROY102 and ROY202) were split for the analysis into small (< 40 g, N = 136) and large individuals (40-200 g, N = 64), and compared with the fast-growth individuals (500-800 g, N = 50). Differences in length and weight among groups were statistically significant.

Allozymes - The 9 enzymatic systems examined resulted in 12 polymorphic loci. Overall tests for Hardy-Weinberg proportions with all polymorphic loci, and for linkage disequilibrium among all loci showed no significant departures from expected values. When observed heterozygosities were compared between growth groups, the group of small individuals presented lower heterozygosities (H_o= 0.192 ± 0.217) than the group of large individuals (H_o= 0.217 ± 0.200), while the highest heterozygosities were observed in the fast-growth individuals (H_o= 0.235 ± 0.225) (Table 2). Differences in observed heterozygosities between

groups were statistically significant ($p< 0.05$). The group of fast-growth individuals presented higher values of observed heterozygosity for most of the loci in comparison with small and large individuals, including *AAT-1**, *GPI-1**, *GPI-2**, *IDHP**, *MPI**, *PGDH** and *PGM** (Table 2). Additionally, polymorphism was also higher in fast-growth individuals (0.692) than in large (0.615) and small (0.539) individuals.

Table 2. *Anguilla anguilla*. Values of observed heterozygosity (H_o), level of polymorphism (P_{95}) and mean number of alleles (MNA) at all allozyme and microsatellite loci for small (<40 g), large (40 - 200 g) and fast-growth (500 - 800 g) individuals. Standard error in parentheses. $*p < 0.05$

Locus	Small	Large	Fast growth	Locus	Slow	Large	Fast growth
Allozymes				Microsatellites			
*AAT-1**	0.051	0.047	0.061	AAN 01	0.761	0.762	0.750
*AAT-2**	0.029	0.000	0.041	AAN 03	0.209	0.227	0.178
*AAT-3**	0.103	0.188	0.102	AAN 05	0.728	1.000	0.778
*ADH**	0.504	0.468	0.490	ARO 063	0.951	0.949	0.905
*GPI-1**	0.267	0.375	0.440	ARO 095	0.899	0.950	0.846
*GPI-2**	0.052	0.094	0.100	ANG 151	0.858	0.818	0.900
*IDDH**	0.431	0.547	0.500				
*IDHP**	0.037	0.094	0.102				
*MDH-2**	0.193	0.328	0.245				
*MPI**	0.140	0.171	0.260				
*PGDH**	0.675	0.492	0.688				
*PGM**	0.007	0.016	0.020				
Mean H_o	0.192*	0.217*	0.235*	Mean H_o	0.711	0.753	0.726
P_{95}	0.539	0.615	0.692	P_{95}	1.000	1.000	1.000
MNA	3.308	3.000	2.769	MNA	18.167	11.333	13.667

In order to correlate genetic variability and growth at the individual level, multi-locus heterozygosity (MLH) was calculated for each individual (Figure 1). When pooling all individuals (combining batches 1 and 2), a highly significant positive association was observed between MLH - length increase ($r= 0.177$; $p= 0.005$) and between MLH - weight increase ($r= 0.164$; $p= 0.009$). Similarly, when the fast-growth individuals were not included in the analysis, a significant correlation was found between MLH - length increase ($r= 0.153$; $p= 0.030$) and MLH - weight increase ($r= 0.143$; $p= 0.044$). In batch 1, the correlations between MLH - length increase ($r= 0.065$; $p= 0.518$) and between MLH - weight increase ($r= 0.066$; $p= 0.517$) were positive but not statistically significant. By contrast, a significant correlation was found in batch 2 between MLH - length increase ($r= 0.240$; $p= 0.016$) and MLH - weight increase ($r= 0.230$; $p= 0.021$).

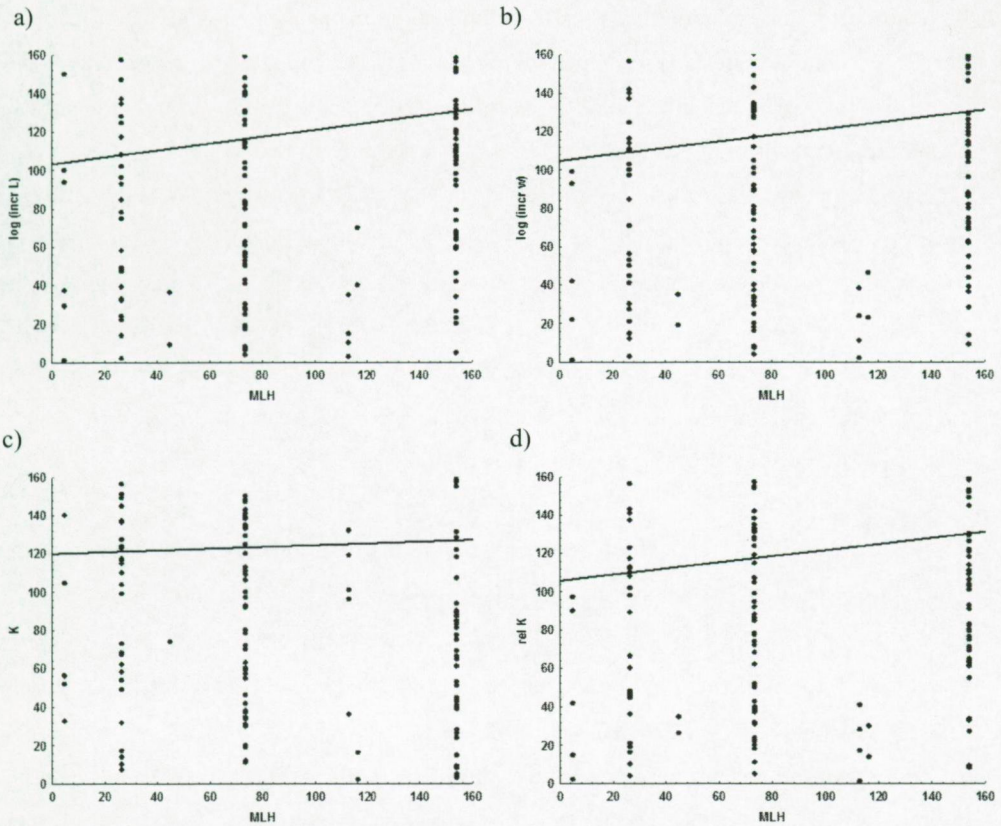


Figure 1. *Anguilla anguilla.* Spearman rank correlation of multi-locus heterozygosity (MLH) versus (a) length (cm) increase (r= 0.177; p= 0.005*), (b) weight (g) increase (r= 0.164; p= 0.009*), (c) condition index (r= 0.044; p= 0.484), and (d) relative condition index at 12 allozyme loci in all individuals (r= 0.157; p= 0.013*).

Multi-locus heterozygosity at allozymes explained 3.1% of the variation in length increase and 3.3% of the variation in weight increase. When mean MLH was calculated for each growth group, slow individuals presented the lowest MLH (0.188 ± 0.087) in comparison with large individuals (0.216 ± 0.102), while the greatest MLH was found in fast-growth individuals (0.229 ± 0.108). When HFCs were tested within growth groups, no significant associations were observed between MLH and growth rate for either small, large or fast growth individuals.

Ricker's condition index (CI) was calculated as $1000(W/L^{3.348})$ for each individual, using the length-weight regression for all samples in order to correct for population effects. The correlation found between MLH - condition factor was positive but not significant when pooling all samples (r= 0.045; p= 0.484), but negative when considering the batches

separately or when excluding fast growth individuals in the analysis (Figure 1). When using the relative condition index (K), significant correlations were found for all pooled individuals ($r= 0.157$; $p= 0.013$) and for batch 2 ($r= 0.215$; $p= 0.032$), and a positive but not significant correlation for batch 1 ($r= 0.063$; $p= 0.537$).

Examination of individual loci suggests that the effect of allozyme heterozygosity on growth is attributed to most of the loci examined. Out of 10 loci (in which all genotypes were sufficiently represented), heterozygotes presented a greater length than homozygotes at 10 loci and a greater weight and relative condition at 9 loci (Table 3). Differences between homozygotes and heterozygotes were maximal at *GPI-1** and *MPI**, with a difference of 5.5 cm in mean length and >70 g in mean weight (70.5 g at *GPI-1**and 80.2 g at *MPI**). These were the only statistically different comparisons ($p< 0.05$).

Microsatellites - Tests for Hardy-Weinberg proportions with all polymorphic loci, and for genotypic disequilibrium among all loci showed no significant departures from expected values. In contrast to allozyme data, the highest heterozygosities were found in large individuals ($H_o= 0.753 \pm 0.276$) in comparison with small ($H_o= 0.711 \pm 0.253$) and fast-growth individuals ($H_o= 0.726 \pm 0.275$); differences were not statistically significant (Table 2). Fast growth individuals only presented a higher (but not significant) heterozygosity at one of the six microsatellite loci examined (ANG 151) in comparison with the other growth groups. Small individuals also presented a higher mean number of alleles (18.167) in comparison with large (11.333) and fast-growth (13.667) individuals.

A small positive effect of microsatellite heterozygosity on growth was observed in most loci examined, although no comparisons were statistically significant. Heterozygotes presented a greater length and weight than homozygotes at 4 out of 6 loci examined and a higher relative condition at 5 loci (Table 3). Differences between heterozygotes and homozygotes were maximal at ARO 095 (4.14 cm in length; 58.6 g in weight) and ANG 151 (3.15 cm in length; 54.1 g in weight). Individual multi-locus heterozygosity (MLH) showed a positive correlation between growth rate (measured as length and weight increase) and heterozygosity at 6 microsatellite loci, although this association was not as strong as found for the allozyme markers (Figure 2).

Table 3. *Anguilla anguilla*. Difference in mean length (L), mean weight (W) and relative condition index (K) values for homozygotes and heterozygotes at ten allozyme and six microsatellite loci. Standard deviation in parentheses. D indicates the sign of difference in all three factors between heterozygotes and homozygotes.

Locus	L (mm)	D	W (g)	D	K	D
Allozymes						
*ADH**						
Homozygotes	315.15		143.02		0.390	
Heterozygotes	320.10	+	158.35	+	0.392	+
*AAT-1**						
Homozygotes	313.32		148.40		0.390	
Heterozygotes	331.07	+	135.48	-	0.396	+
*AAT-3**						
Homozygotes	316.07		148.64		0.391	
Heterozygotes	333.23	+	178.56	+	0.394	+
*GPI-1**						
Homozygotes	300.28		128.39		0.387	
Heterozygotes	354.92	+*	198.92	+*	0.400	+
*GPI-2**						
Homozygotes	315.12		147.69		0.391	
Heterozygotes	349.78	+	190.22	+	0.398	+
*IDDH**						
Homozygotes	310.05		144.29		0.390	
Heterozygotes	327.08	+	156.61	+	0.394	+
*IDHP**						
Homozygotes	312.53		144.66		0.390	
Heterozygotes	364.19	+	203.05	+	0.404	+
*MDH-2**						
Homozygotes	310.24		142.86		0.390	
Heterozygotes	336.67	+	169.03	+	0.394	+
*MPI**						
Homozygotes	307.76		136.71		0.389	
Heterozygotes	362.57	+*	216.95	+*	0.399	+
*PGDH**						
Homozygotes	320.69		141.99		0.394	
Heterozygotes	320.94	+	158.94	+	0.391	-
Microsatellites						
AAN 01						
Homozygotes	346.22		222.45		0.392	
Heterozygotes	340.59	-	198.99	-	0.394	+
AAN 03						
Homozygotes	328.38		186.17		0.390	
Heterozygotes	311.83	-	164.82	-	0.388	-
AAN 05						
Homozygotes	315.64		190.37		0.386	
Heterozygotes	335.13	+	192.93	+	0.393	+
ARO 063						
Homozygotes	341.50		206.22		0.391	
Heterozygotes	350.57	+	215.61	+	0.395	+
ARO 095						
Homozygotes	290.14		132.33		0.384	
Heterozygotes	331.49	+	190.91	+	0.391	+
ANG 151						
Homozygotes	294.19		130.77		0.387	
Heterozygotes	325.67	+	184.85	+	0.390	+

(*) = $p < 0.05$

Positive but not significant correlations were observed between MLH - length increase (r= 0.142; p= 0.070) and MLH - weight increase (r= 0.145; p= 0.063) and when fast-growth individuals were not included in the analysis (MLH - length increase: r= 0.163; p= 0.081; MLH - weight increase: r=0.166; p= 0.076). In batch 1, correlations were positive but not

significant between MLH - length increase (r= 0.237; p= 0.076) and significant between MLH - weight increase (r= 0.264; p= 0.047). In batch 2 correlations were positive but not significant (MLH - length increase: r= 0.037; p= 0.786; MLH - weight increase: r=0.037; p= 0.782). Multi-locus heterozygosity at microsatellite loci explained 1.0% of the variation in length increase and 1.3% of the variation in weight increase. When mean MLH was calculated for each growth group using microsatellite loci, large individuals presented the largest MLH (0.751 ± 0.146) in comparison with fast-growth (0.733 ± 0.166) and slow (0.703 ± 0.158) individuals. No HFCs were observed within growth groups.

Negative associations were observed between MLH and Ricker's condition index in all possible comparisons. Using the relative condition index, positive but not significant correlations were observed for batch 1 (r= 0.117; p= 0.137), batch 2 (r= 0.032; p= 0.815) and when pooling all individuals (r= 0.152; p= 0.052).

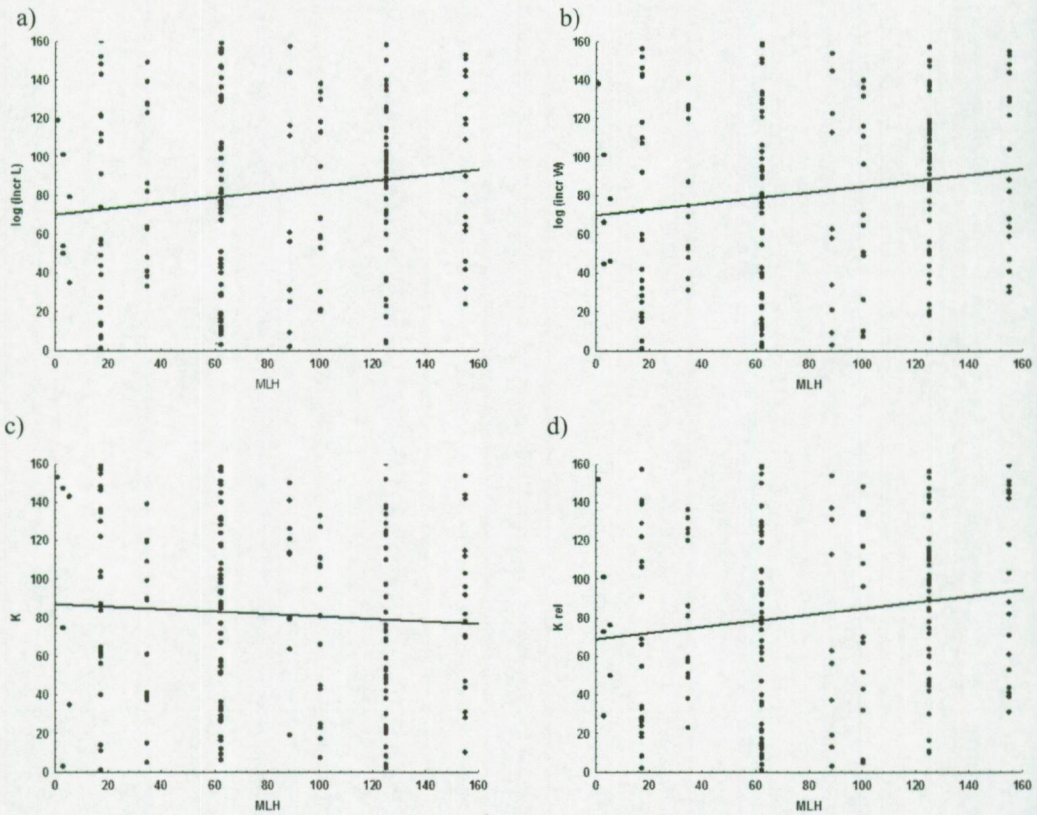


Figure 2. *Anguilla anguilla.* Spearman rank correlation of multi-locus heterozygosity (MLH) versus (a) length (cm) increase (r= 0.142; p= 0.070), (b) weight (g) increase (r= 0.145; p= 0.063), (c) condition index (r= -0.061; p= 0.433), and (d) relative condition index at 6 microsatellite loci in all individuals (r= 0.152; p= 0.052).

The logarithmic transformation of mean d^2 was negatively correlated to growth rate (Figure 3). Negative associations were found between mean d^2 and length increase (r= -0.045; p= 0.557), weight increase (r= -0.050; p= 0.537), condition index (r= -0.058; p= 0.478) and relative condition index (r= -0.055; p= 0.494), respectively. Similar negative correlations were obtained when homozygous loci in each individual were excluded (outbreeding mean d^2) or when mean d^2 was standardized at each locus by the variance of d^2 (mean $d^2_{var\ (d2)}$).

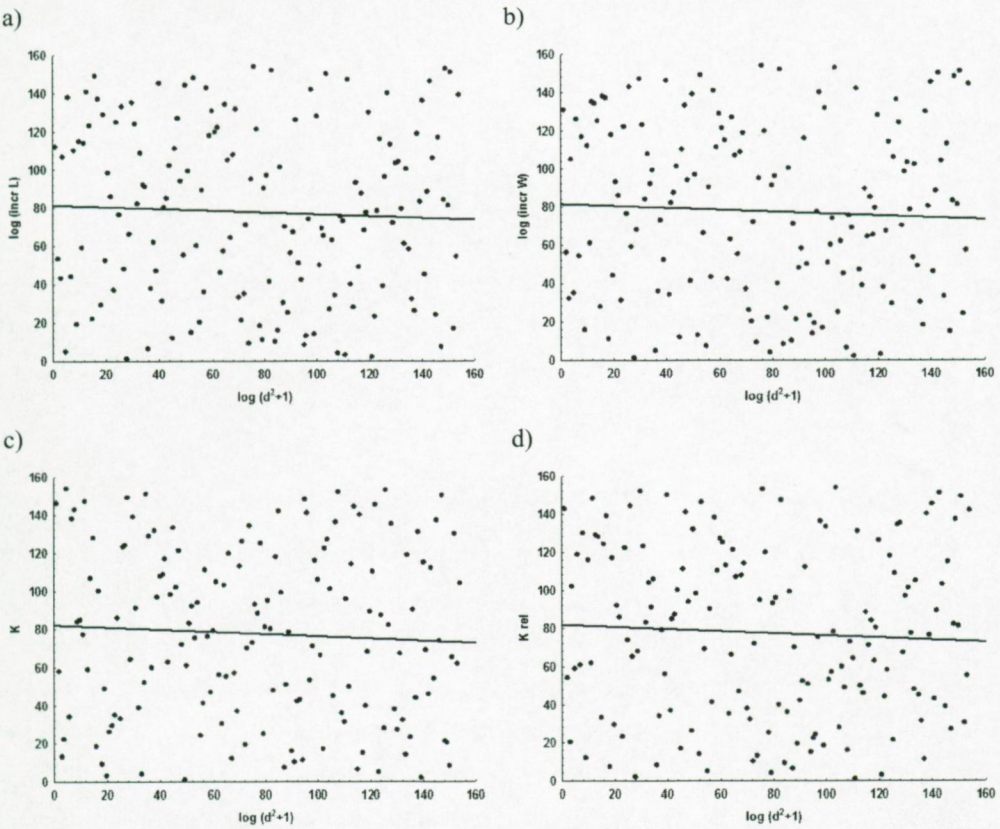


Figure 3. *Anguilla anguilla*. Regression of mean d^2 versus (a) length (cm) increase (r= -0.045; p= 0.557), (b) weight (g) increase (r= -0.050; p= 0.537), (c) condition index (r= -0.058; p= 0.478), and (d) relative condition index at 6 microsatellite loci in all individuals (r= -0.055; p= 0.494).

When testing the association between genetic variation at allozymes and microsatellite markers, a non significant negative correlation was found between individual MLH at allozyme loci and individual MLH at microsatellite loci (Figure 4).

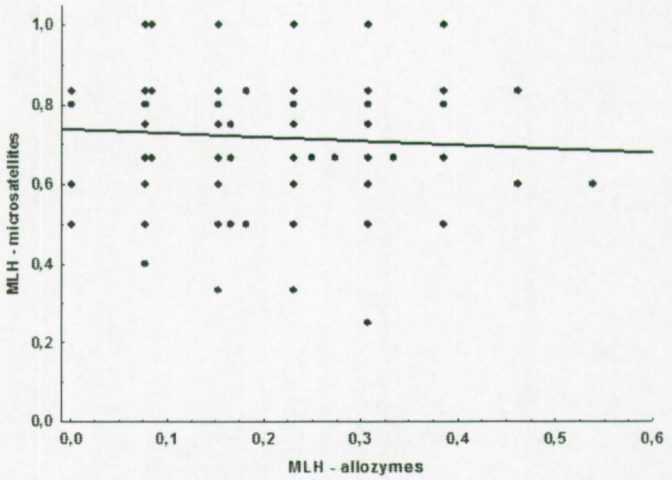


Figure 4. *Anguilla anguilla.* Regression of multi-locus (MLH) at 12 allozyme loci versus MLH at 6 microsatellite loci in all individuals (r = 0.062; p= 0.431).

DISCUSSION

Evidence for heterozygosity-fitness correlation in European eel

We clearly show a positive correlation between heterozygosity at allozyme loci and growth rate; more heterozygous individuals presented a higher length and weight increase and a greater relative condition index in two separate batches of farmed European eel. The stronger correlations observed in batch 2 in comparison with batch 1 might be related to their greater variation in length and weight; differences in growth rate should be easier to detect in such conditions. At microsatellite loci, a general pattern was observed that individuals with a larger growth rate mostly presented higher heterozygosities. Correlations were positive but not significant, and HFCs at microsatellites cannot be considered to be as strong as HFCs at allozymes. Multi-locus heterozygosity at allozymes also explained more variation in length and weight increase (3%) than MLH at microsatellites (1%) in multiple regressions. As heterozygosity in European eel explains less than 5% of the individual variation in growth rate, the large fraction of unexplained variation in growth rate is partitioned between environmental effects, genetic factors other than the loci studied and epistatic interactions. Similar correlation values have been reported in previous HFC studies, usually accounting for a small proportion of the observed phenotypic variance (3 - 6%) (Britten, 1996). A meta-

analysis of correlations between phenotypic variation and genetic variation revealed that the strength of the associations are generally weak (mean $r < 0.10$), with a mean r of 0.0274 for MLH and 0.0156 for mean d^2, respectively (Coltman & Slate 2003). Examining HFCs in fish, multi-locus allozyme heterozygosity explained more than 10% of the total variance of the condition factor in rainbow trout (*Oncorhynchus mykiss*) (Thelen & Allendorf 2001). In the positive correlations between genetic variation (both microsatellite heterozygosity and d^2) and four reproductive fitness-related traits in chinook salmon (*Oncorhynchus tshawytscha*), regressions explained between 6 - 8% of the variance (Heath *et al.* 2002).

Our results show that heterozygosity at allozyme and microsatellite loci, albeit scored for an unequal number of loci, has a different effect on the individual phenotype and supports the view that natural selection does not affect allozymes and microsatellites similarly. A negative correlation was observed between individual MLH at allozymes and individual MLH at microsatellites (Figure 4), implicating that a high level of genetic variability at allozyme loci does not necessarily correspond with a high genetic variation at microsatellite loci. In fish, Thelen & Allendorf (2001) examined the relationship between MLH and condition factor at 10 allozyme and 10 microsatellite loci in 217 farmed rainbow trout (*O. mykiss*). While more heterozygous individuals at allozyme loci showed a significantly higher condition factor, increased heterozygosity at microsatellite loci was not associated with a higher condition. More recently, Borrell *et al.* (2004) reported a positive correlation between heterozygosity and fluctuating asymmetry, length and weight in two samples of Atlantic salmon (*Salmo salar*) with different timings of first active feeding at six allozyme loci but not at eight microsatellite loci.

Positive correlations were observed in our study between MLH and all growth measures except condition. Weight is highly variable among eel populations; optimal feeding conditions may cause an eel's weight to be twice that of another eel of equal length (Tesch 2003). This is especially valid in aquaculture and might explain the lack of correlation between MLH and condition observed in our study. On the other hand, relative condition mostly reflects changes in weight regardless of changes in length. Hence the positive association between MLH and relative condition is a direct consequence of the positive correlation observed between MLH and weight.

Regarding the performance of MLH and mean d^2, our results are concordant with the meta-analysis of published and unpublished HFCs carried out by Coltman and Slate (2003), where correlations reported for mean d^2 were smaller than those reported for MLH. Contrasting with the positive correlation between MLH and growth in our study, the negative

association between mean d^2 and growth in European eel (Figure 3) suggests that mean d^2 might not be an adequate estimator of genetic variability. Positive associations between mean d^2 and fitness traits have been reported in species with high inbreeding coefficients (Coltman *et al.*, 1998; Coulson *et al.*, 1998), where mean d^2 refers to the relatedness of individuals and not genetic variation. In the absence of inbreeding, which is typical for species with large population sizes, high migration rates and lack of population substructuring, mean d^2 does not necessarily reflect genetic variation. This explains the negative relation in our study between mean d^2 and growth rate.

Support for the direct overdominance hypothesis

A prediction of the direct overdominance hypothesis is that only heterozygosity scored at selection-sensitive markers should correlate with fitness-traits, while no relationship should be expected at neutral markers. This contrasts with the associative overdominance hypothesis where HFCs are not specific to the type of genetic marker used. In our study, both markers showed positive correlations between the degree of heterozygosity and growth rate, but only significantly so at allozymes. Similarly, fast-growth individuals presented significantly higher heterozygosities than the rest of individuals at allozymes but not at microsatellites. Allozymes produced a more significant and consistent association than microsatellites, thus providing evidence for the direct overdominance hypothesis as an explanation for the HFCs observed. Nevertheless, it must be taken into account that although microsatellites are usually considered as evolutionarily neutral DNA markers (Schlötterer & Wiehe, 1999), recent evidence indicates otherwise and at least some microsatellites are functionally important and may not be neutral (Li *et al.*, 2002). Pogson & Zouros (1994) proposed that under the associative overdominance hypothesis re-calculation of MLH combining both allozymes and microsatellites loci would result in stronger HFCs than each set of markers independently. In our study, correlations with the combined set of 18 loci were identical to the ones obtained with allozyme data, with an apparent lack of effect of microsatellite loci.

Selection affecting some of the allozyme loci would explain the stronger HFCs at allozymes in comparison with microsatellites, and the lack of correlation between MLH at allozymes and MLH at microsatellites. At allozymes, more heterozygous individuals present an intrinsically higher fitness due to increased biochemical efficiency in comparison with more homozygous individuals (Mitton, 1993, 1997). Model organisms like *Drosophila melanogaster* and *Fundulus heteroclitus* have provided clear evidence for selection acting on

enzyme polymorphisms in metabolic genes (Eanes, 1999). Koehn *et al.* (1988) reported significant effects of heterozygosity on growth at enzymes involved in metabolic functions such as protein catabolism or glycolysis, while genes without significant effect on growth rate code for enzymes with other miscellaneous functions including the pentose shunt, redox balance or digestion. Metabolic responses to selection are essentially multi-locus in nature (reviewed in Eanes, 1999). The effects of allozyme heterozygosity on growth are the result of many loci, although not all contribute equally (Pogson & Zouros, 1994; Thelen & Allendorf 2001). In the scallop *Placopecten magellanicus*, the mean shell length in heterozygotes was larger than in homozygotes at 8 loci, but only significant at 2 loci (Pogson & Zouros, 1994). In our study, positive differences in growth between homozygotes and heterozygotes were observed at almost all loci. Statistically significant values were only obtained when comparing differences in length and weight increase at *GPI** and *MPI**. Both loci are involved in important metabolic functions: mannose-6-phosphate isomerase (MPI) is a pre-glycolytic enzyme, which supplies carbon skeletons to glycolysis. Glucose phosphate isomerase (GPI) is a main-line glycolytic enzyme that catalyses the reversible interconversion of d-fructose-6-phosphate and d-glucose-6-phosphate. By contrast, the smallest differences between homozygotes and heterozygotes in growth rate were observed at phosphogluconate dehydrogenase (PGDH), which codes for an enzyme with a secondary metabolic function (pentose shunt). Since PGDH is not involved in protein catabolism or glycolysis, it would play a smaller role in growth.

Alternative hypothesis: associative overdominance

Associative overdominance could provide an explanation for the HFCs in this study if we consider that allozyme loci have a higher chance than microsatellites to be in linkage disequilibrium with fitness loci. A greater mutation rate of microsatellites would cause homozygous genotypes to present identical alleles by state (homoplasy) and not by descent, and therefore are less likely to reflect homozygosity at linked fitness loci. Alternatively, allozyme and microsatellite loci might not have the same distribution in the genome. If allozyme loci are located in richer gene regions and microsatellite loci in poorer gene regions, allozymes would be more likely to be in linkage disequilibrium with fitness loci (Thelen & Allendorf 2001). Little information is available on the distribution of genes in the European eel, although linkage maps in for example salmonids (pink salmon, Lindner *et al.* 2000; brown trout, Sakamoto *et al.* 2000) suggest that allozyme and microsatellite loci are found at

similar genomic locations. Tóth *et al.* (2000) reported that despite microsatellites being less abundant in exons than in noncoding regions, microsatellite distribution in introns and intergenic regions is similar and differs only in the abundance of certain triplets.

The main mechanisms explaining HFCs detected by neutral markers relate to inbreeding due to low effective population sizes or linkage disequilibrium caused by bottleneck/founder events followed by rapid population expansion (Hansson & Westerberg 2002). Glass eel recruitment is estimated at about 2×10^9 individuals per year, although mortality is close to 100% in some areas (Dekker 2000). As in many marine fish species, anthropogenic impacts (fisheries overexploitation, pollution, habitat loss and migration barriers) cause populations to decline. Since the 1980's, a steadily downward trend has been observed in recruitment of glass eel arriving to the European continent, which is affecting or will shortly affect the continental (yellow and silver eel) stock (ICES 2003). Using a Bayesian approach to infer demographic parameters from microsatellite data, Wirth & Bernatchez (2003) suggested a contemporary effective population size of about $5 \times 10^3 - 10^4$ eels. Despite the population of European eel being possibly at an historical minimum, it would remain large enough not to be affected by inbreeding.

Conclusions and future directions

Our results provide sound evidence for a heterozygosity-fitness correlation in farmed European eel individuals. Multi-locus heterozygosity is positively correlated with growth, so that more heterozygous individuals at allozyme loci (and to a lesser extent microsatellite loci) show a significantly higher length and weight increase and a higher condition index in comparison with more homozygous individuals. The HFCs observed could be explained by an effect of either direct allozyme overdominance or associative overdominance.

HFCs are expected to decrease or disappear with age since growth and survival differences are maximal early in life (David, 1998). It has been proposed that early and late growth may be controlled by different sets of genes (Vaugh *et al.*, 1999). In our study, individuals will be kept in the farm for another year, which will allow us to check for time consistency of the observed HFCs. We intend to estimate genotype-specific survival and possible heterozygosity-viability correlations by analyzing individuals that died after handling and parasite infection, and the influence of grading (early splitting of individuals in size classes) in the association MLH-growth.

ACKNOWLEDGMENTS

We thank G. Pogson and three anonymous reviewers for their useful comments on the manuscript. We also thank V. van Ginneken and all the personnel at Royaal BV who assisted us with sampling. Research was funded by the EU contract EELREP (Q5RS-2001-01836). G.E. Maes received a PhD fellowship from the I.W.T. (Institute for the Promotion of Innovation by Science and Technology in Flanders). J.M. Pujolar received a postdoctoral fellowship from the Ministerio de Educación, Cultura y Deporte (Spain). Both authors were supported by the EU contract EELREP (Q5RS-2001-01836).

GENERAL DISCUSSION

This thesis assessed the evolutionary consequences of a catadromous life strategy on the genetic architecture of European eel. The main findings were organized into three distinctive parts: (1) first, a molecular toolbox for species discrimination was developed before intra-specific analyses could be performed. After having detected a substantial amount of genetic leakage between both North-Atlantic eel species, (2) a detailed intra-specific analysis revealed a pattern of Isolation-by-Distance (IBD) in adult eels. When increasing the resolution by using highly variable markers on many geographical and temporal samples, this pattern seemed to be elusive and unstable in time. But, by standardizing the sampling scheme and specifically targeting recruiting juveniles, a double pattern of temporal variance in genetic structure was observed. A large scale signal of Isolation-By-Time (IBT) was detected between yearly spawning cohorts, likely induced by a large variance in parental contribution, and small scale pattern of genetic patchiness, caused by variance in adult reproductive success. The decline in recruitment within the European eel may be due to a combination of high reproductive variance and anthropogenic stress. On its turn this may be associated with a reduced genetic diversity within the entire species. The relationship between genomic diversity and fitness is known; but in the last two chapters we show an association between individual genetic diversity and heavy-metal bioaccumulation on the one hand and growth rate on the other hand. A further decline of the population size of eel might well bring the whole species in an extinction spiral, through the synergetic effects of anthropogenic disturbances (such as overfishing, pollution, habitat degradation and disease) and a shift in ocean dynamics, reducing the predictability for future spawners.

1. Observations

Evolutionary consequences of a catadromous life-strategy on speciation and hybridisation in eels

All 15 freshwater eel species are highly adapted to oceanic gyral systems to complete their life history. The ancestor of the genus *Anguilla* was most probably a fully marine species, which evolved to facultative catadromy and entered the rivers in search for food (Tsukamoto *et al.*, 2002). The high trophic level of freshwater rivers gave a selective advantage to catadromous individuals, resulting in the present almost obligate catadromous life-strategy (Tsukamoto *et al.*, 2002; Tesch, 2003). Speciation has likely occurred in two ways, namely through distributional range expansions of the feeding habitat within the same gyral system (Introduction, Figure 22, Type I) or by dispersing to another adequate oceanic gyre (Introduction, Figure 22, Type II) (Tsukamoto *et al.*, 2002; Ishikawa *et al.*, 2004). Through adult and larval dispersal, eels have been able to colonize and adapt their life strategy to large parts of the world's oceans, as long as a cyclonic current pattern was functioning. This pattern also fits within the member vagrant hypothesis, where members stay in their gyral system, while vagrants may or may not end up in a new suitable environment (Sinclair, 1988).

The speciation time is still under discussion; the genus *Anguilla* is considered to have experienced a recent evolutive radiation (Avise, 2003, Minegishi *et al.*, 2004). Morphological identification of all species is a difficult task, requiring precise molecular methods to identify the species status of morphologically indistinguishable life stages or to detect natural hybridisation and anthropogenic translocations (Watanabe *et al.*, 2004). Microsatellite flanking regions are thought to be highly conserved in fish taxa, enabling their application in other species within or outside the source family (Rico *et al.*, 1996). However, microsatellite based phylogenetic reconstructions remain dubious due to allele size homoplasy (Estoup *et al.*, 2002). Species identification using multi-locus genotypes may suffer less from this phenomenon, when based on moderately variable markers. In **Chapter 1**, we evaluated the degree of conservation of microsatellite flanking regions and the level of polymorphism in relation to phylogenetic distance in four eel species (*Anguilla anguilla, A. rostrata, A. japonica* and *A. marmorata*). Using multiplex PCR reactions developed for the first two taxa, we assessed the discrimination power of an individual based assignment method to differentiate all four species without prior information. Detection and classification of each species and assignment of randomly sampled individuals to pre-defined species were

performed with high confidence (> 90% and > 95%, respectively). Our results demonstrate the highly conserved nature of microsatellites and their level of polymorphism in *Anguilla* species, and the power to discriminate between locus-specific and population dynamic effects on genetic variability. Although an inverse relationship was found between genetic diversity and differentiation estimates due to homoplasy, assignment proved to be superior to multivariate and distance based approaches. The method allows for the rapid screening of the species status of elvers and adults using four loci, including the detection of natural hybridization (see **Chapter 2**) or anthropogenic mixing between internationally traded species.

A direct application of the results of Chapter 1 involved the reassessment of the hypothesis of hybridisation between the sympatric North Atlantic European eel (*A. anguilla* L.) and American eel (*A. rostrata*) (**Chapter 2**). A small percentage of fish with a low number of vertebrae has been found in Icelandic individuals, pointing to the possible presence of hybrids (Avise *et al.*, 1990). Both species share the same spawning region with a significant overlap in spawning period (McCleave, 1993; Tesch, 2003). Due to the catadromous life strategy of both species, their reproductive isolation is highly dependent on the strength of pre- and postzygotic barriers. Due to the long transoceanic migrations of the European eel, genetic leakage between both species may occur (Ishikawa *et al.*, 2004). By testing the joint distribution of microsatellite markers and vertebral counts, I first characterized the genetic variability and differentiation between both species in populations throughout Europe and America. I then screened several Icelandic populations for introgressive hybridisation of European eel with American eel. Icelandic diversity values were intermediate between both species. Results from mainly moderately variable microsatellite loci, specially selected to avoid homoplasy, yielded a high genetic differentiation between both species (F_{ST} = 0.14, R_{ST} = 0.11; $p < 0.001$), congruent with earlier mitochondrial DNA studies (Avise *et al.*, 1986). Subsequent multivariate and individual based assignment tests separated both species with high confidence (> 95% assignment score). Classical and a model-based Bayesian individual assignment tests detected a total admixture within Iceland of 11-15%, mainly composed of F_1 hybrids (6.3%) and pure *A. rostrata* (3.8%) individuals, which was much higher than previous values based on mitochondrial DNA and allozymes (Avise *et al.*, 1990). The total number of vertebrae was lower in Iceland and co-varied strongly with the admixture coefficient, indicating the taxonomic stability of this meristic trait. Our results suggest a narrow tension zone, with asymmetric introgressive hybridisation towards the European eel. Due to the low number of

F_1 hybrids and genomic linkage disequilibrium in Icelandic populations, this hybrid zone is thought to be a dispersal-selection zone (Hewitt & Barton, 1995). The incomplete genetic isolation of North Atlantic eels spawning sympatrically affected additional locations in Europe with cryptic American-like genomes. The primary or secondary nature of this tension zone is difficult to infer. Under the influence of glaciations, two groups may have formed in the Atlantic, followed by assortative mating and low overlap in spawning time/region. After the Last Glacial Maximum (LGM) both species expanded their range again, with the European eel exhibiting the longest catadromous migration loop of all anguillids (Tsukamoto *et al.*, 2002). Whether both species were at some point completely separated from each other is not known, but sympatric divergence with gene flow has been shown to occur more often than expected (Mallet, 2005). Reinforcement, which is expected to be the strongest in sympatric zones, may safeguard the differential oceanic migration of both species, as hybrids in highly migratory species are known to be maladaptive due to intermediate migrational cues between both parental species (Berthold, 1988). In the North Atlantic eels, without strong pre-zygotic barriers or selection against fitness, hybridization would lead to a homogenisation of the gene pools and the loss of both species.

In brief, in this first part, a tool to detect future anthropogenic translocations or natural hybridisation events between morphological similar eel species was developed. We also showed a significant introgression of the American eel genome within the European eel, which is most likely due to a tension zone in the Sargasso Sea to safeguard species integrity. Due to the stock decline of several *Anguilla* species and the strong influences of global change on atmospheric and current pattern fluctuations, translocations of foreign eel species or natural hybridisation between formerly isolated species are likely to occur more often than thought. These two studies enable the critical assessment of such processes in other *Anguilla* species.

Evolutionary consequences of a catadromous life strategy on the spatio-temporal genetic structure of eels

Life history traits of highly vagile marine species, such as adult reproductive success and the potential for larval dispersal are driven by oceanographic and climatic factors. Nevertheless, marine organisms have the potential for restricted dispersal in time and space. Considering the ability of leptocephali to actively control their migration to the American or European continent, it may be possible that the large natural range of the European eel and the

substantial variance in spawning time induce spatio-temporal reproductive isolation between cohorts (Tesch, 2003).

In **Chapter 3,** the genetic variability and structure of the European eel in adult populations throughout Europe using 15 allozymic loci was analysed. Seven sites were sampled on a latitudinal gradient across the natural continental range, extending from southern France to southern Norway. Although populations were weakly differentiated (G_{ST} = 0.014, F_{ST} = 0.002), which is not surprising considering the high dispersal capability of the European eel, a significant geographical cline was detected at two loci (*IDH-1** and *GPI-1**) and more importantly genetic distances (D_{CE}) were concordant with geographical coastal distances. Three groups could be distinguished, namely Northern Europe, Western Europe and the Mediterranean Sea. Several explanations were proposed for this pattern, such as 1) a pattern of Isolation-by-Distance (IBD, as recently detected with microsatellites), 2) temporal reproductive separation, 3) post-larval selective forces, 4) secondary contact between formerly separated groups or 5) any combination of the previous options.

Marine fishes clearly exhibit a very subtle genetic structure, experience large variance in reproductive success and show a low signal/noise ratio, so that the signal of differentiation might easily be missed or misinterpreted by a single point sampling strategy (Waples, 1998). In the case of the European eel, sampling bias may play a significant role in genetic studies due to its protracted spawning season, wide distribution and extremely high genetic variability (Daemen *et al*, 2001; Wirth & Bernatchez, 2001, Tesch, 2003). It is only likely if spawning subpopulations are spatially and/or temporally separated, followed by non-random larval dispersal. Previous genetic studies on allozymes (Chapter 3) and microsatellites (Daemen *et al.*, 2001; Wirth & Bernatchez, 2001) did not include temporal replicates to test for temporal stability of the genetic structure. We hypothesized that temporal genetic variation may play an important role in explaining the spatial structure reported earlier on. I tested this by increasing the texture of geographical sampling and by including temporal replicates from various sampling locations. Overall genetic differentiation among samples was low, highly significant (F_{ST} = 0.0014; $p < 0.01$) and comparable to earlier studies based on microsatellites (Daemen *et al.*, 2001; Wirth & Bernatchez, 2001). On the other hand, hierarchical analyses revealed no significant inter-location genetic heterogeneity and hence no IBD anymore. Instead, genetic variation among temporal samples within sites clearly exceeded the geographical component. These results emphasized the importance of temporal replication when assessing population structure of marine fish species and prompted for a closer look to the temporal genetic variability of spawning cohorts.

Temporal variance in allele frequencies can be attributed to two factors. First, as proposed in **Chapter 4**, the European eel may have a much lower effective population size (N_e) than expected from census counts (N_c) (Dekker, 2003; Dannewitz *et al.*, 2005). Due to variance in adult reproductive success in each spawning season, N_e is usually much smaller than N_c and may lead to strong fluctuations in allele frequencies in each generation. Unfortunately, the calculation of the effective population size remains complex in eel, as adults mature at variable ages (from 6 to 40 years), making a reliable calculation of N_e highly speculative with the present samples. The average yearly recruitment in the European eel is about 2 billion larvae, whereas only 9 million adults may escape to spawn. This 99% population size reduction was not observed at genetic markers in Chapter 4, as diversity values were very similar between adults and juveniles. The mixed age structure of adults may be the reason, but the adult continental pool is still decreasing, moving the problem to 10-15 years later. The analysis of historical sample may show more clearly the population decrease, which started some 40 years ago (Dekker, 2003). On a smaller scale, genetic patchiness between cohorts under influence of environmental conditions is expected to have a similar effect on allele frequencies, if samples are not taken at the same time or continuously (Hedgecock, 1994; Waples, 1998). Secondly, temporal variance may originate from the sampling of temporally separated cohorts that remained separated during their trans-oceanic migrations as adults or larvae. To detect such pattern, the sampling strategy has to be standardized within and between years (Waples, 1998, Hendry & Day, 2005). Due to a sampling strategy incorporating various life stages (glass eel, yellow eel and silver eel) in Chapter 4, temporal differentiation was detected but Isolation-by-Time between yearly cohorts remained difficult to detect, unless adults were aged. In **Chapter 5**, we wanted to separate the forces shaping the genetic composition of recruiting juveniles of the European eel. By controlling for geographical variation through sampling at six sites, we assessed temporal variation during three consecutive years and tested for Isolation-by-Time between spawning cohorts. Using morphometric, as well as 12 polymorphic allozyme and eight variable microsatellite loci, we found again that genetic differentiation was low (F_{ST} = 0.2% - 0.9%) between cohorts but consistent with a pattern of Isolation-by-Time (IBT) (Hendry & Day, 2005). Nevertheless, we could not detect a seasonal IBT pattern, but only a large scale inter-annual differentiation and a small scale influence of genetic patchiness.

When reflecting on the results of these three studies, we may propose a possible scenario for the population structure of European eel. Its population dynamics may be governed by a double pattern of variance in genetic composition. Due to the variable age at maturity in adult

eels and their long trans-oceanic migration, the genetic constitution of spawning adult may differ significantly between years. When adding a seasonal variance in reproductive success of adult (unpatterned) within a year with bad oceanic conditions, a pattern of IBT can be created without a heritable component. A seasoneal IBT would hev been difficult to detect, due to the very low numbers of recruits of late spawners (that experienced poor conditions in the Sargasso Sea) entering the rivers. The consistency and stability of this IBT remains to be tested in spawning or migrating aged adults to confirm the random/non-random nature of such pattern in eel.

Evolutionary consequences of a catadromous life-strategy on heterozygosity-fitness correlations

The genetic diversity characteristics of a species are essential to its long-term survival, as heterozygosity is positively related to fitness and the global pool of genetic information represents the 'blueprint' for life (Frankham *et al.*, 2002). Organisms exhibiting low levels of genomic variability may suffer from a reduced fitness, through the negative effect of inbreeding depression and genetic load of lethal allelic variants. The European eel stock is declining rapidly, due to overfishing, pollution, parasites, migration barriers, habitat degradation and oceanic conditions. Current recruitment levels of the European eel are strongly correlated to the North-Atlantic Oscillation Index (NAOI) (Knights, 2003), which is in accordance with the results in Chapter 5. They indicate that genetic variability and differentiation estimates may be associated with (un)favourable climatic conditions. This stresses the importance of integrating oceanic knowledge in eel management strategies (Knights, 2003). In unfavourable years, a strong variance in reproductive success may lead to a loss of genetic variation within the entire species, resulting in a lower effective population size. If fitness is associated with genetic variability, such decrease might endanger the species even more. This has been for a long time a conundrum in marine taxa: can adaptation to anthropogenic factors be proven? The catadromous life-strategy of eels provides the potential for evolutionary studies on the effect of genetic variability on fitness components, due to the lack of an interfering genetic background of recruiting glass eels at a small scale and the absence of local adaptation in freshwater resident populations. The correlation between individual heterozygosity and fitness-related traits such as growth, survival, fecundity or developmental stability has never been shown before in eel. Such heterozygosity-fitness correlations (HFCs) have important consequences for a species, as in declining populations

they might trigger an extinction spiral, which accelerates even more the decline. Through the negative effect of genetic load and inbreeding, marine organisms may exhibit very abrupt population crashes. The cause-effect relationship is difficult to provide as a population decrease influences genetic variability, influencing on its turn the population stability and fitness. In chapter 6 and 7, I assessed whether the genetic background of European eel is linked with two fitness traits, namely detoxification success and growth.

In **Chapter 6**, the relationship between heavy metal bioaccumulation, fitness (condition) and genetic variability in the European eel was studied. A significant negative correlation between the pollution load of heavy metals and condition was observed, suggesting an impact of pollution on the health of subadult eels. The production of, for example, methallothioneins to capture heavy metals represents a heavy cost, possibly lowering the individual fitness and condition. In general, we observed a reduced genetic variability in strongly polluted eels, as well as a negative correlation between the level of bioaccumulation and allozymatic multi-locus heterozygosity (MLH). Microsatellite genetic variability did not show any pollution related differences, suggesting a differential response at metabolic enzymes and possibly direct overdominance of heterozygous individuals.

In **Chapter 7,** evidence for a positive correlation between genetic variability and growth rate at 12 allozyme loci in European eel was provided. More heterozygous individuals showed a significantly higher length and weight increase and an above average condition index in comparison to more homozygous individuals. To a lesser extent, six microsatellite loci showed a similar pattern, with positive but not significant correlations between heterozygosity and growth rate.

Summarizing both studies, there was strong evidence for Heterozygosity-Fitness Correlations, that might be explained either by an effect of direct allozyme overdominance or associative overdominance. Selection affecting some of the allozyme loci would explain the greater strength of the HFCs found at allozymes in comparison with microsatellites, and the lack of correlation between MLH at allozymes and MLH at microsatellites. Associative overdominance (where allozyme loci are merely acting as neutral markers of closely linked fitness loci) might provide an alternative explanation for the HFCs. If allozyme loci were located in gene richer regions and microsatellite loci in gene poorer regions, allozymes would be more likely to be in linkage disequilibrium with fitness loci (Thelen & Allendorf, 2001). Little information is available on the distribution of genes in the European eel, although linkage maps in for example zebrafish (Singer *et al.*, 2002) and salmonids (Atlantic salmon, Moen *et al.*, 2004) suggest that allozyme and microsatellite loci are found at similar genomic

locations. Such results suggest that individual heterozygosity plays an important role in survival, detoxification and growth, but that this may be only true at quantitative trait loci, including metabolic allozymes. Microsatellites are thought to be selectively neutral and will only show HFCs in specific conditions, such as in small populations or if they are linked to adaptive traits. HFCs do however have consequences on the population structure and persistence of the European eel. The positive consequence of the eel's catadromous life history is that locally polluted rivers will only have a low impact on the entire population, due to the lack of spatial genetic structure at the local level. Nevertheless, selection on each generation will erode genetic variability in a different way locally, possibly slowly decreasing overall genetic variability. Differential selective pressures might induce differences between spawning cohorts in time and space, possibly increasing the temporal differentiation pattern observed in Chapter 5. Similarly, fast growing individuals will mature early and may be more heterozygous than slow growing individuals. Unfortunately, eutrophic systems producing large females with much fat reserve are also the most polluted habitats. During spawning migration, pollutants will strongly decrease the fitness of such individuals, weakening the whole populations. If population size decreases even more together with genetic variability, less fit individuals will be left over for the long spawning, weakening even more the complete species.

In the next figure, I summarize the findings of each chapter. We found indication for a tension zone between the North-Atlantic eel species, likely to have been maintained by a strong dispersal-selection mechanism and confined through reinforcement. The spatio-temporal genetic structure within species is very subtle, but is likely governed by a pattern of Isolation-by-Time between years, dependent on the adult genetic constitution, and a high variance in adult reproductive success during seasonal spawning periods, dependent on oceanic and continental conditions (Figure 1). Finally, a relation between genetic diversity and fitness was observed, pointing to the possibility of additional lower fitness due to population size decrease.

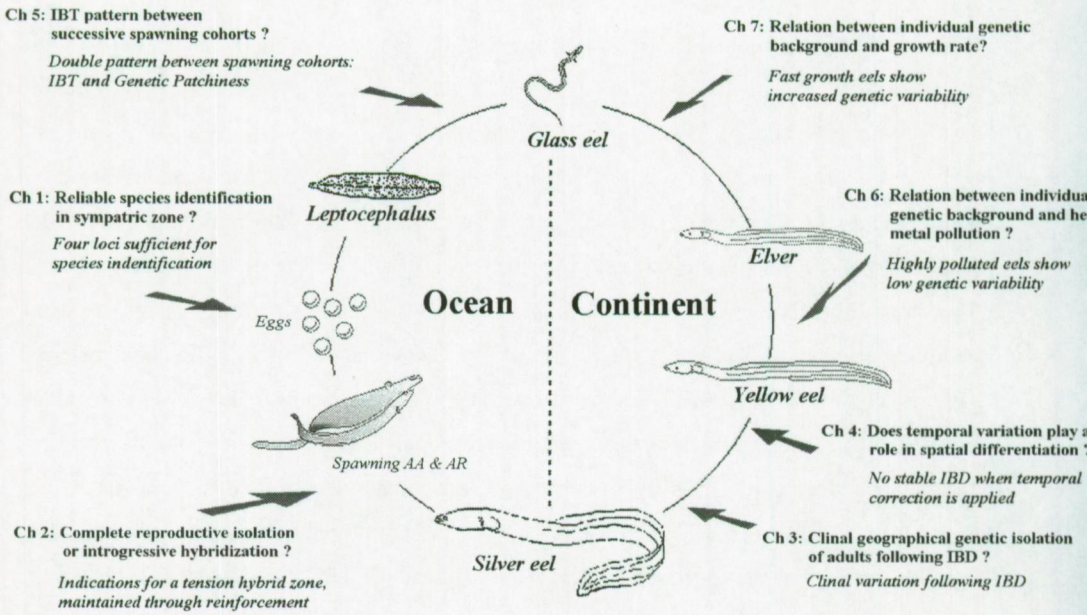


Figure 1: Life cycle of *Anguilla anguilla*, showing the initial questions asked in this study and their respective answers for each chapter.

2. A possible scenario for the past and current evolution of the European eel

Freshwater eel (*Anguilla* spp.) belong to the Elopomorpha, a group of fishes all exhibiting a leptocephalus larval stage and exclusively inhabiting the ocean. The genus *Anguilla* consists of 15 species worldwide, originating from one ancestor having adopted a catadromous life-strategy to take advantage of the high trophic composition of the freshwater environment (Tsukamoto *et al.*, 2002). All eel species exhibit this peculiar life-strategy and are adapted to an oceanic gyral system to complete their life-cycle. Through dispersal and circum-equatorial currents the ancestor of the North-Atlantic eels colonized the Atlantic before the closure of either the Thetys Sea (10 myA) or the Panama Isthmus (3 myA). During my research on *A. anguilla*, I realized that the catadromous life-strategy of eel had several important consequences on its evolution and current genetic architecture. The ancestor of *A. anguilla* and *A. rostrata* was most likely continuously distributed along the coasts of North-America and Europe, but under influence of Pleistocene glaciations and the advance of the ice sheets,

two groups were formed due to the inhospitable habitat in the polar regions. Although initial spawning may have been nearly panmictic, sympatric speciation may have developed as eels from each side of the Atlantic started to spawn assortatively at slightly different sites and times. Subsequent glaciations may have influenced the effective population size of both species, due to a lower productivity of the Sargasso Sea and a lower velocity of the Gulf Stream during the Last Glacial Maximum (LGM) (Wirth & Bernatchez, 2003). This might have lead to periods of population reduction and expansion, where spawning time and place repeatedly had to be readjusted.

The catadromous life strategy of the European eel has several genetic implications on species integrity and hybridisation dynamics. If environmental conditions become less stable or predictable than before (known as global change) or endocrine disruption of the spawning stimulus influences the timing of reproduction, both North-Atlantic species might start to increase their overlap in spawning season and might hybridise secondarily. On the other side to maintain species integrity other mechanisms, such as spatio-temporal distance and strong selection against hybrids may minimize the number of hybrids in a primary zone. Field evidence (Chapter 2) cannot provide an answer to the question of the nature of the tension zone. Conversely, when two species have been separated for millions of years, but put in contact by human translocations, the consequences are unknown. The European eel has been introduced in Japanese eel waters and might endanger its evolutionary persistence, as experimental hybrids are viable and European eel has been reported to co-migrate (Okamura *et al.*, 2000). A molecular tool enabling the detection of such hybrids is of key importance (Chapter 1). Similar to both North-Atlantic species choosing actively their migration path, the hypothesis was raised that within each species a similar pattern could arise. Considering the size of the largest migration loop in the European eel, northern populations might preferentially reproduce with conspecifics. A similar argument may be raised for Southern/Mediterranean populations.

Various studies (Chapter 3 inclusive) suggested a pattern of Isolation-by-Distance in the European eel, due to spatio-temporally separated groups at the spawning site, but such pattern seemed highly unstable when replicated in time. To reconcile both views, the catadromous life-strategy of eels was re-analysed thoroughly. There are two possible explanations for divergence of eel populations through a shift of the migration loop: (a) Populations may expand their feeding habitat and start to spawn at a slightly different time or site (the North-Atlantic eels) or (b) populations may recolonize a new gyral system and found an independent migration-loop (*A. marmorata*) (Ishikawa *et al.*, 2004). None of such patterns fully apply to

the intra-specific divergence in the European eel. A third scenario may nevertheless provide an answer for our results. Differentially spawning populations might differ in spawning time instead of spawning place (Hendry & Day, 2005); such pattern requires nevertheless a strong heritable component to maintain a stable separation. In Chapter 5, the analysis of recruiting cohorts over several years provided evidence for an IBT pattern, but most obviously between years and not within the spawning season. A double pattern was proposed to explain the high variance in genetic composition of recruits: a broad scale "Isolation-by-Time" of spawning cohorts, possibly as a consequence of large differences in adult spawning cohort constitution, and a smaller scale variance in adult reproductive success (genetic patchiness) among seasonally separated cohorts, most likely influenced by strong oceanic and climatic influences. The next figure summarizes our findings and emphasizes the need for additional temporal analyses over a longer period to test for long-term stability of the pattern observed.

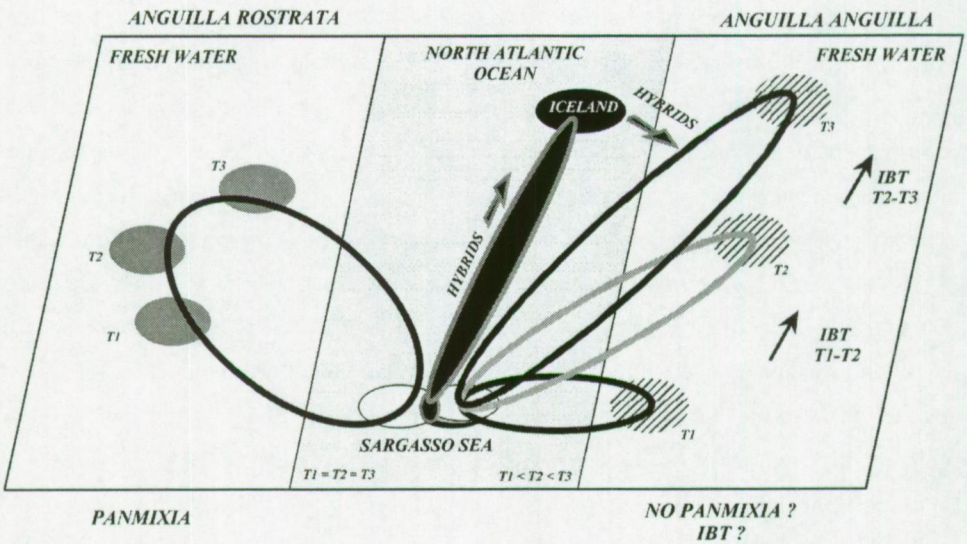


Figure 2: Diagram showing a scenario for the contemporary genetic structure of both North-Atlantic species based on the occurrence of one (*A. rostrata*) or several (*A. anguilla*) temporally separated migration loops, with a limited amount of hybridisation between both species (adapted from Tsukamoto *et al.*, 2002).

3. Implications

The threats on the sustainability of the European eels originate from several sides; both anthropogenic and oceanic factors influence the evolutionary potential and repercussions are visible on the genetic architecture of marine or continental populations. The future of the species is in jeopardy, unless man integrates oceanic knowledge into management issues and takes more care to avoid far reaching translocations of foreign species.

Through inter-continental trading of live eel, man has endangered the evolutionary potential and integrity of the whole genus *Anguilla*. Several species have become established outside their natural range. The consequences of possible hybridization between two unrelated species is unknown, but considering the importance of a perfect match between migration time, spawning and food supply, hybridization is expected to be maladaptive and an evolutionary dead end for both species. More research should focus on the dynamics of natural and man-induce hybridization to fully understand the consequences of gene flow on their life-cycle.

The fisheries status of European eel has been considered outside biological safe limits. Most stock rescue management issues focus on the continental phase, such as lowering fisheries pressure, enhancing eel migration potential and restocking of juveniles (provided they originate from wild catch and have not been enriched with slow growing animals from aquaculture farms). Although such approaches will certainly be effective on the adult population in rivers, the reproductive potential is believed to be low due to other factors. High mortality is observed in metabolically weak migrating adults under influence of the swimbladder parasite *Anguillicola crassus* and due to the accumulation of pollutants in fat tissue, needed for the long trans-oceanic migration (Van Ginneken *et al.*, 2004). Fluctuating oceanic conditions also lower drastically reproductive success of adults and recruitment level (Knights, 2003). The current genetic data show no clear geographical separation in stocks, but temporally differentiated spawning waves under the influence of oceanic conditions. Such pattern renders management issues complex. We propose the full integration of oceanic knowledge into management decisions, so that in addition to continental measures, an assessment is made of the climatic and oceanographic conditions of future years, influencing larval survival and recruitment.

Pollution in eels is a crucial problem due to their high fat content, which is used for the long trans-oceanic migration. Our results show that pollution affects resident populations and that the genetic background of an individual plays a role in detoxification. Highly

heterozygous individuals were less polluted and also showed a higher growth rate in aquaculture (Pujolar *et al.*, 2005). The loss of genetic diversity through the synergy of overfishing, pollution and fluctuating oceanic condition might thus additionally impact the entire species through the generation of less fit individuals, with a lower reproductive success. Artificial reproduction is an outcome for relaxing the pressure on natural populations, but results are still highly unsatisfying. Another important unsuspected problem is the stocking of aquaculture "left-overs"; consisting of individuals with a low growth rate, thus low expected fitness. If the fit individuals are sold, the "worst" genomes are restocked in rivers to produce adult spawners. This will inevitably decrease the reproductive success in the Sargasso Sea even more.

When we integrate our genetic results in a broad scale study of the reproductive potential of European eel (European project EELREP), we observed that genetic background is just one component out of many influences. In figure 3, a summary is given of the findings, where many factors influence the silvering process, the migration performance and the maturation performance.

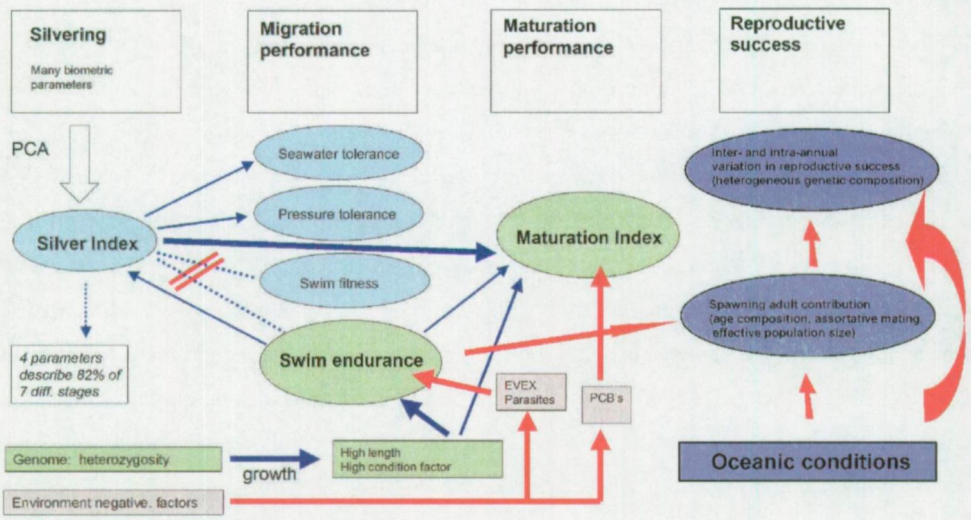


Figure 3: Synthesis of the possible physiological and genomic factors influencing the reproductive potential in eel (adapted from the project EELREP, G. van den Thillart, pers. comm.).

The main results were a wide age at maturity range in female eels silvering, a high mortality in polluted, EVEX-virus or *Anguillicolla crassus* infected eels in swimming eels after 1000-2000 km, an onset of final maturation due to pressure and a complicated response to hormonal treatments (compared to Japanese eel). The genomic heterozygosity may be of importance to explain survival and growth, both characters crucial for reproductive success. Additionally, strong variance in reproductive success under influence of oceanic conditions reduces even more successful spawning and larval survival in the ocean.

4. Future directions

There are three directions for future research on eels, namely the history of the genome, the analysis of adaptive genetic variation and the management of sustainable resources.

In first instance, the present thesis looked at the short-term spatio-temporal genetic structure of *Anguilla anguilla*. Although indication of Isolation-by-Time and oceanic influences on genetic variability were found, long-term time series are much more reliable to make such inferences. A first step is the use of aged adults, so that back calculations till 30-40 years ago can be performed. More importantly, to assess the influence of heavy fisheries and yearly/decadal fluctuating oceanic conditions, the analysis of historical material of 100 years and more is needed. This is now possible due to newly developed genetic techniques for ancient DNA and will enable the reliable calculation of a pre- and post industrial fishery genetic population size. This knowledge is of crucial importance to define sound management issues.

Indications of hybridisation between sympatric eel species were found in the North-Atlantic. Most genetic studies on anguillids are performed using mitochondrial DNA, making such analysis of hybridisation impossible. The complexity of the phylogeny of eels even using the complete mitochondrial genome (Minegishi *et al.*, 2004) might well be due to historical admixture and introgression events. It is known that hybridisation can be of importance to colonize new habitats, enabling new adaptations to oceanic gyral systems. If dispersal and migrations are highly adaptive and heritable, such hybridisation might well be needed to break this migration-loop and enable speciation. The analysis of co-occurring species using nuclear markers might unveil a very different phylogeny and help the detection of anthropogenic introductions.

Although the intraspecific genetic structure is very subtle in many eel species, neutral genetic variation might well underestimate adaptive variation over a broad environmental

range. The development and study of novel markers such as Expressed Sequence Tags (ESTs) and Single Nucleotide Polymorphisms (SNPs) would enable the detection of many adaptive variation sites, to screen for environmentally dependent fitness traits. SNPs are considered the markers of the future, due to their unambiguous scoring (compared to microsatellites), short fragment size (suitable for ancient DNA), neutral/adaptive characteristics and uniform polymorphism across the genome.

Pressure on the eels is mostly due to the lack of artificial reproduction. Since 30 years, researchers have been unable to produce economically profitable quantity of eels in aquaculture. Oceanic knowledge might help defining better hatching protocols, but candidate genes for fast growth or immunity against parasites from ESTs could already reduce the need for huge amount of recruiting glass eels.

The European eel has been studied for over hundred years and hypotheses concerning its population structure were tested using newly developed techniques every time they appeared. Nevertheless, the black box remains tightly closed for researchers. Many factors of its catadromous life-strategy increase the chance of panmixia, such as the variable age at maturity, the highly mixed spawning cohorts, the protracted spawning migration, the sex biased latitudinal dispersal and the unpredictability of oceanic conditions. Nevertheless, several active components induce the chance for population divergence, such as assortative mating behaviour, the segregation of both North-Atlantic species in the Gulf Stream, active trans-oceanic larval migration, the presence of hybrids mainly in Iceland and the extremely large migration loop of European eel compared to other species. In this thesis, it became clear that a geographical component, if existing, is almost invisible. On the other hand, genetic data supports strong temporal variation between and within year as a consequence of large variance in adult contribution and reproductive success. Oceanic forces are likely to represent the main actor in the observed temporal variation. The present climatic oscillations combined with the significance of oceanic forces in marine species prompts to the urgent assessment of temporal stability of the European eel stock, combining genetic, population dynamics and oceanic data. Only by tracking migrating adults and genetic monitoring their offspring through time will enable the reliable assessment of the factors influencing the population structure of the European eel. We believe the present study has provided additional knowledge on the population structure of eel, namely by showing the occurrence of a hybrid zone between the two sympatric North-Atlantic eel species, by the detection of an Isolation-by-Time pattern among yearly spawning cohorts and by presenting evidence for a relation between heterozygosity and fitness.

Generation after generation, scientists have dedicated their time and energy to the study of the European eel. Although a long way has been covered since Aristotle's theory of spontaneous generation in eels, the endless quest to unveil the fascinating life cycle of this mysterious creature will ultimately take us back to the Sargasso Sea, where everything started...

The truth is out there!

SUMMARY

Marine organisms usually exhibit a high genetic diversity, a subtle population structure and a low level of genetic differentiation, compared to freshwater organisms. The subtle genetic differences in time and space reflect the continuity of the marine environment. Marine organisms experience a wide range of intrinsic and extrinsic influences during their life cycle, which considerably impact their biological population size and genetic population structure. Furthermore, genetic variability is crucial for the survival of organisms as it enables evolution while maintaining fitness. Marine species however have a high genetic load, affecting the population even more during a population decline or bottleneck. The European eel *Anguilla anguilla* (Anguillidae; Teleostei), although inhabiting fresh- and saltwater, represents no exception. Its spawning habitat in the Sargasso Sea and extensive migrations across the North Atlantic Ocean qualify it fully as a marine species. This thesis describes the multiple evolutionary consequences of the catadromous life-strategy on the genetic structure of the European eel.

Recent data based on microsatellite markers show a subtle genetic structure in the European eel following an Isolation-by-Distance (IBD) pattern. But since genetic introgression from the American eel into the European eel has been suggested in the North Atlantic Ocean, reliable tests were developed to define the species status of the European eel. In this first part the interspecific conservation of a set of microsatellites was tested on other *Anguilla* taxa and the power of species discrimination was assessed. We then applied this knowledge by screening Icelandic and European samples for introgression of American eel. Indications of unequal but restricted hybridisation were detected, likely maintained through selection against hybrids and the preservation of migrational cues.

In the second part, the genetic variability and differentiation between various glass and silver eel populations was compared over a broad geographical range (Iceland to Morocco; Spain to Turkey) with temporal replications. In the first instance a pattern of Isolation-by-Distance was detected in adult populations using allozyme markers. Following a more extended geographical sampling, the temporal stability of this pattern could not be confirmed; the temporal differentiation between populations clearly exceeded the geographical component. By sampling recruiting glass eels over a three-year period, a stronger genetic differentiation was found between temporally separated cohorts. Inter-annual differentiation was much higher than the geographical differentiation. The population genetic structure of eel is likely determined by a double process: (1) a large scale pattern of Isolation-by-Time (IBT) among spawning cohorts, and (2) a smaller scale variance in adult reproductive success (genetic patchiness) among seasonally separated cohorts, most likely originating from oceanic and climatic influences.

In the third part, the relation between multi-locus heterozygosity (MLH) and fitness components was studied. If an association exists between genetic variability and fitness traits, it is even more important to maintain the population size of European eel. The catastrophic decline of the European eel might be the consequence of an accelerated loss of genetic diversity, with extinction as possible outcome. This hypothesis was tested in a polluted natural environment and in an eel farm. Eel from three Belgian drainage basins were screened for fitness, heavy metal bioaccumulation and genetic variation. There was a strong negative correlation between MLH and bioaccumulation in highly polluted eels. In a second study,

aquacultured eel were screened for fitness and genetic variation; MLH was correlated to growth rate. In both studies, this effect was mainly attributed to metabolical enzymes, important in the energy cycle, which points to the importance of polymorphism at functional markers. Hence, reduced genetic variability may enforce the loss of fitness in eel.

In summary we allege that 1) genetic introgression occurs from the American eel into the European eel (mainly in Iceland); 2) the geographical pattern of genetic differentiation is much lower than temporal variation between years and life stages; and reproductively isolated cohorts show a pattern of Isolation-by-Time; and 3) that the individual genetic pattern of eel is important for survival in situations of either stress or competition. Our results have consequences for the management of the fishery and aquaculture of eel. Glass and silver eel fishery should be limited, river migration routes should be improved and pollution should be urgently dealt with. Glass eel transfer between river catchments and even between continents should be avoided. Despite its common name of "freshwater eel", the European eel is a marine species that should be managed on a global European scale and not locally, as is the case now. Eel aquaculture should put more effort in the efficient growing of glass eels, by improving artificial breeding. In the future, more attention should be given to long-term oceanic influences on population stability of Anguillids and on the analysis of adult cohorts to test the stability of the Isolation-by-Time pattern.

SAMENVATTING

Mariene organismen vertonen over het algemeen een meer homogene populatiestructuur en geringere differentiatie t.o.v. zoetwater organismen. Subtiele genetische verschillen in tijd en ruimte worden nochtans waargenomen. De katadrome paling (*Anguilla anguilla* L.) staat tijdens zijn lange levenscyclus onder invloed van heel wat externe invloeden, met belangrijke gevolgen voor zijn biologische populatiegrootte (sterke daling sinds 1960) en zijn genetische populatiestructuur. Verder is genetische variabiliteit cruciaal voor de overleving van organismen; ze bepaalt de mogelijkheid om in de toekomst te kunnen evolueren en voldoende fitness te behouden. Mariene soorten bezitten echter een hoge genetische last die bij een daling in populatiegrootte (flessenhals), de populatie des te sterker kan treffen. Deze thesis beschrijft de veelzijdige evolutionaire consequenties van een katadrome levensstrategie op de genetische structuur van de Europese paling. De resultaten vallen in drie luiken uiteen:

Gepubliceerde gegevens gebaseerd op microsatelliet DNA toonden aan dat de Europese paling een subtiele genetische structuur vertoonde volgens een isolatie door afstand model. Maar aangezien vermoed wordt dat er genetische introgressie gebeurt vanuit de Amerikaanse paling, en dit vooral in Noord-Europa, werden eerst betrouwbare testen ontwikkeld om de soortstatus van Europese individuen te karakteriseren. De interspecifieke toepasbaarheid van onze microsatelliet merkers werd getest op verwante palingsoorten om de statistische kracht voor soortenonderscheid te testen. Vervolgens werd deze kennis toegepast op Noord-Atlantische palingsoorten door IJslandse en Europese stalen te toetsen op introgressie door de Amerikaanse paling. Indicaties voor ongelijke, maar beperkte hybridisatie werden gevonden. De lage graad van hybridisatie wordt waarschijnlijk in stand gehouden door middel van selectie tegen hybriden en het behoud van migratierichting.

In een tweede luik werd de genetische variabiliteit en differentiatie tussen verschillende glasaal- en zilveraalpopulaties vergeleken over een uitgestrekte geografische schaal (van IJsland tot Marokko; Spanje tot Turkije) met temporele replicatie. Vooreerst werd aan de hand van allozymatische merkers een patroon van isolatie-door-afstand (IDA)gevonden in de Europese volwassen populaties. Na een uitgebreide staalname in tijd en ruimte, kon de stabiliteit van dit patroon niet aangetoond worden, waarbij het grootste deel van de genetische differentiatie tussen populaties te wijten was aan temporele verschillen. Door het bemonsteren van glasaal cohortes over een driejarige periode, werd uiteindelijk een sterkere genetische differentiatie gevonden tussen temporeel gescheiden stalen. Inter-annuële verschillen waren veel hoger dan ruimtelijke verschillen. De populatiegenetische structuur van paling wordt mogelijks bepaald door een dubbel proces: (1) een patroon van isolatie-door-tijd (IDT) van reproducerende cohorten op grote schaal en (2) een verschil in reproductief succes van volwassenen, waarschijnlijk het gevolg van de heersende oceanische en klimatologische condities.

In een derde luik werd de relatie tussen multi-locus heterozygositeit (MLH) en fitness componenten nagegaan. Indien er een verband bestaat tussen genetische variabiliteit en fitness kenmerken, is het nog belangrijker om de populatiegrootte van paling te bewaren. De sterke achteruitgang van de palingpopulatie zou hierdoor nog kunnen versneld worden, met uitsterven tot gevolg. In door zware metalen vervuilde palingen werd een duidelijke negatieve correlatie gevonden tussen MLH en bioaccumulatie. Bij palingen uit een vetmesterij werd een positieve correlatie gevonden tussen MLH en groeiratio. In beide studies werd dit effect

hoofdzakelijk op metabolische enzymen waargenomen, die instaan voor de energiecyclus, waarbij polymorfisme op functionele merkers van cruciaal belang blijkt te zijn.

Samenvattend kunnen we stellen dat 1) genetische introgressie voorkomt vanuit de Amerikaanse paling in Europa (vooral in IJsland); 2) het ruimtelijke patroon van genetische differentiatie veel lager is dan temporele variatie tussen jaren en levensstadia, en reproducerende cohorten een isolatie-door-tijd patroon vertonen en dat 3) het individuele genetisch patroon van paling van belang is voor overleving in situaties van stress of competitie. De gevolgen van dit onderzoek situeren zich op het vlak van de visserij en de aquacultuur. De visserij op jonge glasaal en zilverpaling moet teruggeschroefd, rivieren moeten vrije toegang bieden tot trekkende paling en de vervuiling van rivierslib moet dringend krachtdadiger aangepakt worden. Transfers van jonge paling tussen rivierbekkens en zelfs continenten zijn uit den boze. Ondanks zijn bijnaam als "rivierpaling", is de Europese paling een mariene soort die op globale Europese schaal beheerd dient te worden en niet lokaal zoals nu het geval is. De paling aquacultuur zelf moet efficiënter jonge glasaal opkweken en liefst op termijn overgaan tot kweek van paling in plaats van jonge dieren af te vangen. Enkel dan is het voortbestaan van paling mogelijk. In de toekomst, zou meer aandacht besteedt moeten worden aan oceanische factoren die de populatiestructuur van Anguillidae kunnen beïnvloeden en aan de analyse van volwassen dieren met gekende leeftijd, om de stabiliteit van het IDT patroon na te gaan.

POPULAIRE SAMENVATTING

Paling werd tot voor kort als een algemene soort beschouwd in Vlaanderen. Dat is veranderd doordat vandaag de dag de aankomst van jonge paling (glasaal genaamd) in de lente minder dan 1% bedraagt van de aantallen in de jaren zeventig. Bovendien leeft de paling in zodanig vervuilde wateren, dat de palinghengelaar wettelijk zijn eigen vangst niet meer mag opeten. Het gaat dus helemaal niet goed met één van de meest merkwaardige vissen uit de Lage Landen.

Paling paait in de verre Sargassozee (ergens tussen Bermuda en Florida) en sterft kort nadien. De jonge larven (bladwilglarven genaamd) beginnen aan een tocht van zes tot negen maand in de Golfstroom en Noord-Atlantische Drift naar het Europese continent. Ze voeden zich met kleine wiertjes en bacteriën (plankton genaamd) tot ze ongeveer tien centimeter groot zijn. Wanneer ze de rand van het continent bereiken (continentaal plat genaamd) ondergaan ze een metamorfose tot glasaal, die de vorm van de welbekende paling aannemen. Palingen ontstaan dus niet uit de modder zoals in de Oudheid en Middeleeuwen werd aangenomen. De kleine glasaal zwemt richting riviermonden en trekt de rivieren op. Dat fenomeen was jaarlijks goed te volgen aan de sluizen van de Grote Rivieren bij volle of nieuwe maan. Eens paling zich in de rivier bevindt voedt hij zich eerst met kleine organismen die in de bodem leven om later over te schakelen op grotere prooien zoals vissen en zelfs soortgenoten. De nauwe associatie met de bodem en het hoge vetgehalte van het spierweefsel zijn de oorzaak van de systematische opstapeling van een brede waaier aan vervuilende stoffen, inclusief de zeer schadelijke gechloreerde organische koolwaterstoffen. Na zes (mannetjes) tot tien (vrouwtjes) jaar ondergaat de zogenaamde gele paling een nieuwe metamorfose. De ogen worden groter, de darm verkleint en de voortplantingsorganen worden groter. Men spreekt dan van zilverpaling die zijn eerste en laatste lange tocht (ca 6000 km) naar de Sargasso Zee kan aanvatten.

Uit de literatuur is bekend dat paling een subtiele genetische structuur vertoont, net zoals vele andere mariene organismen. Anders gezegd, door de menging van de oceanen, is de kans klein dat sterk verschillende populaties van paling zich handhaven. De paaigronden in de Sargassozee lopen te gemakkelijk in elkaar over zodat paling als één enkele populatie kan beschouwd worden. Behalve dan in IJsland, waar er zich een speciale situatie voordoet. De populaties van paling bestaan daar uit kruisingen van de Europese paling (*Anguilla anguilla*) en de Amerikaanse paling (*Anguilla rostrata*). Dit doctoraat toont aan dat dergelijke kruisingen in ongeveer 10-15% van de gevallen voorkomen en dat hybriden soms tot in Europa worden waargenomen.

Maar er is meer met de subtiele genetische verschillen in de oceaan. Doctor Gregory Maes toont aan dat de weinige genetische verschillen hun oorzaak vinden in jaarlijkse samenstelling van de groepen nieuwe aankomers die het Europees continent bereiken. Anders gezegd, de overlevers van de zes tot negen maanden lange transatlantische race verschillen sterk van jaar tot jaar. Dat is gekend in visserijjargon als de "gepaste – foute" koppeling tussen het voedsel (plankton) en de rover (de paling). Dergelijk fenomeen is sterk gevoelig aan de productiviteit van de zee en dus ook aan de grillen en periodiciteit van het klimaat. Bovendien bestaat het vermoeden dat de voortplanting in de Sargassozee toch een subtiel verschil mogelijk maakt, namelijk door lichte tijdsverschillen in het moment van paaien.

Vervolgens heeft doctor Gregory Maes in samenwerking met doctor Marti Pujolar onderzocht of grote palingen al dan niet genetisch een stapje voor hebben. Daar zulke experimenten niet kunnen uitgevoerd worden in de natuur, werd uitgeweken naar een Nederlandse palingkwekerij. Gedurende twee jaar werd de groei van paling, oorspronkelijk aangekocht als glasaal, opgevolgd. Na één jaar bleken de snelle groeiers inderdaad genetisch meer variabel (en dus superieur). Na twee jaar was het fenomeen nog waar te nemen, hoewel opmerkelijk afgezwakt. Tenslotte werden zwaar vervuilde palingen genetisch onderzocht en bleek dat genetisch meer variabele individuen minder polluenten opstapelden dan genetisch arme dieren.

De gevolgen van dit onderzoek situeren zich op het vlak van de visserij en de aquacultuur. De ineenstorting van het palingbestand is spijtig genoeg vandaag een zekerheid . Een internationale vergadering van palingonderzoekers stelde daar een speciale verklaring (lees noodkreet) voor op in Québec (Canada) in 2003. Maar de kans is groot dat binnenkort ook het genetische patrimonium bedreigd wordt. Dus moeten alle negatieve tussenkomsten van de mens op het duurzame voortbestaan van de paling (en talrijke andere levende organismen) binnen een redelijke termijn weggewerkt worden. De visserij op jonge glasaal en zilverpaling moet teruggeschroefd, rivieren moeten vrije toegang bieden tot trekkende paling en de vervuiling van rivierslib moet dringend krachtdadiger aangepakt worden. Transfers van jonge paling tussen rivierbekkens en zelfs continenten (60% van de glasaalexport is bestemd voor ZO Azië) zijn uit den boze. Ondanks zijn bijnaam als "rivierpaling", is de Europese paling een mariene soort die op globaal Europese schaal beheerd dient te worden en niet lokaal zoals nu het geval is. De palingaquacultuur zelf moet efficiënter jonge glasaal opkweken en liefst op termijn overgaan tot kweek van paling ipv jonge dieren af te vangen. Enkel dan is het voortbestaan van paling mogelijk.

RÉSUMÉ

En général, les organismes marins présentent une structure de populations plus homogène et par conséquent une différenciation génétique moindre par rapport aux organismes d'eau douce. Toutefois, de subtiles différences génétiques spatio-temporelles ont été récemment observées. L'anguille catadrome (*Anguilla anguilla* L.) est, au cours de son cycle de vie, sous l'influence de nombreuses forces externes dont les conséquences peuvent être importantes pour la taille biologique et génétique des populations (forte chute depuis 1960). De plus, la diversité génétique est cruciale pour la survie des organismes ; elle détermine la possibilité d'évoluer dans le futur tout en gardant suffisamment de condition (fitness). Les organismes marins possèdent néanmoins un grand fardeau génétique qui affectera beaucoup plus une population décroissante qu'une population stable ou en évolution. Cette thèse décrit les conséquences évolutionnaires multiples de la stratégie de vie catadrome sur la structure génétique de l'anguille européenne. Trois points principaux ont été abordés au cours de ce travail :

Dans un premier temps, deux études de microsatellites (séquences d'ADN) ont démontré que l'anguille européenne présente une structure génétique subtile conforme à un modèle d'isolation par distance (IBD). Toutefois, étant donné les fortes présomptions d'introgression génétique entre les anguilles américaines et européennes en Europe (surtout au Nord), des tests fiables ont du être dévelopés pour déterminer avec certitude l'origine des individus européens. L'application interspécifique d'un set de microsatellite a d'abord été testé sur des espèces d'anguilles apparentées. Puis, ces connaissances ont été appliquées sur les anguilles Nord-Atlantiques par l'analyse d'échantillons islandais et Européens, et la détection d'introgression possible entre l'anguille américaine et européenne sur ces sites. Des indications d'hybridisation inégale mais limitée ont été trouvées, probablement due à une forte séléction contre les hybrides et à l'importance de la migration transatlantique des deux espèces.

Dans un deuxième volet, la variabilité et différentiation génétique entre diverses populations de civelles et d'anguilles argentées ont été comparées sur une échelle géographique étendue (de l'Islande au Maroc ; de l'Espagne à la Turquie) en abordant une approche spatio-temporelle. Tout d'abord, un modèle d'isolation par distance a été observé entre populations européennes adultes en utilisant les marqueurs allozymiques. Après un échantillonage spatio-temporel plus approfondi, la stabilité de ce modèle n'a pas pu être confirmée, car la majorité de la différentiation génétique était liée aux différences temporelles et non géographiques. L'échantillonage de cohortes de civelles sur une période de trois ans a permis de démontrer une différentiation plus prononcée entre les échantillons temporels. Les différences inter-annuelles étaient nettement plus manifestes que les différences géographiques. La structure de population génétique de l'anguille peut donc se résumer par un double processus : (1) un modèle à grande échelle d'isolation dans le temps (IBT) des cohortes se reproduisant dans la mer des Sargasses et (2) une différence du succès reproducteur des adultes, probablement lié aux conditions océaniques et climatologiques.

Dans un troisième volet, la relation entre l'hétérozygotie multi-locus (MLH) et les composants de fitness a été examinée. Un déclin de population diminue de facon significative la variabilité génétique de l'éspèce, et peut avoir des consequences sur sa survie si la MLH est positivement corrélée à la survie de l'éspèce. Le déclin du stock de l'anguille, déjà très

prononcé, pourrait de ce fait être accéléré, avec pour possible conséquence, l'extinction totale de l'espèce à long terme. Chez les anguilles présentent dans des milieux fortement pollués, une corrélation négative a été observée entre la MLH et le niveau de bioaccumulation des polluants. Une corrélation positive a été observée entre la MLH et le taux de croissance chez les anguilles d'aquaculture. Dans les deux études, cet effet est principalement observé pour des enzymes métaboliques, cruciales pour le cycle d'énergie de l'organisme, indicant l'importance du polymorphisme génétique dans les marqueurs fonctionnels.

En résumé, nous pouvons conclure que (1) il existe des traces d'introgression entre l'anguille américaine et l'anguille européenne en Europe (surtout en Islande) ; (2) Le modèle géographique de différentiation génétique influence nettement moins la structure génétique de l'anguille sur son aire de répartition que la variation temporelle observée entre années et stades de vie, les cohortes reproductrices présentant un modèle d'isolation dans le temps ; (3) Les caractéristiques génétiques d'un individu ont une grande importance pour la survie de l'espèce dans des situations de stress et de compétition. Les résultats de ce travail peuvent servir de référence dans de nombreux domaines tels que ceux des pêcheries et de l'aquaculture, et doivent être intégrés dans la gestion des stocks d'anguille, aussi bien sauvages que domestiques. La pêche aux civelles et à l'anguille argentée doit être restreinte. La modification des cours d'eau due aux activités anthropiques (barrage par example) limite la migration des anguilles dans les rivières et doit être limitée pour la sauvegarde de l'espèce. De même, la contamination des cours d'eau par les polluants issus de l'industrie peut être la cause de modification de la structure génétique des populations d'anguille et doit être, par conséquent, considérée comme nuisible pour l'espèce. L'existence de structure génétique au sein des différentes rivières étudiées souligne l'influence néfaste que peuvent avoir les transports de civelles d'une rivière à une autre, qui sont donc à éviter. De plus, malgré sont surnom "d'anguille d'eau douce", l'anguille est un poisson marin, qui doit être géré sur une échelle européenne globale et non pas locale comme aujourd'hui. Le développement des techniques d'aquaculture de l'anguille doit être facilité, notamment pour un développement plus efficace des civelles, mais également afin de pouvoir passer à la reproduction artificielle. Ceci pourrait à long terme, réduire les pressions anthropiques sur l'espèce. Pour l'avenir de l'espèce, il faut maintenant se concentrer d'avantage sur les facteurs océaniques qui influencent la structure des populations des anguillidés et sur l'analyse d'adultes dont l'âge est connu, pour vérifier la stabilité de l'IBT chez l'anguille.

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