Biological Journal of the Linnean Society, 2002, **77**, 509–521. With 4 figures

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

G. E. MAES* and F. A. M. VOLCKAERT

Katholieke Universiteit Leuven, Laboratory of Aquatic Ecology, Ch. de Bériotstraat, 32, B-3000 Leuven, Belgium

Received 10 December 2001; accepted for publication 13 August 2002

The genetic variability and structure of the European eel (*Anguilla anguilla* L.) in populations throughout Europe was reassessed using 15 allozymic loci, seven 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.

ADDITIONAL KEYWORDS: allozymes – Atlantic Ocean – genetic variation – marine organisms – population genetics – selection – spatial structure.

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

*Corresponding author.
E-mail: gregory.maes@bio.kuleuven.ac.be

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 *et al.*, 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 & Bernatchez (2001). The objective of this study was two-fold: 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 *et al.*, 1970; Yahyaoui *et al.*, 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 and 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. The samples were centrifuged for 10 min at 13 000 r.p.m. (10 000*g*) at

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 (Burrishoole)	Ireland (EI)	IRL	53°55′N; 09°55′W	23/09/1997	S	50
Den Oever (IJsselmeer)	the Netherlands (NL)	NE1	53°01′N; 05°13′E	01/10/1997	S (m)	50 (m)
Den Oever (IJsselmeer)	the Netherlands (NL)	NE2	52°27′N; 05°17′E	29/10/1997	S (f)	50 (f)
Pleurtuit (Frémur)	France (F) (Atlantic Ocean)	FR1	48°34′N; 02°03′W	09/1997	Y + S	26
Pila (Po river)	Italy (I)	ITA	44°54′N; 12°22′E	04/1999	Y + S	28
Sète (Lagune de Thau)	France (F) (Mediterranean Sea)	FR2	43°24′N; 03°41′E	12/1998	Y + S	50

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) SGE and the procedure of Richardson *et al*. (1986) CAGE.

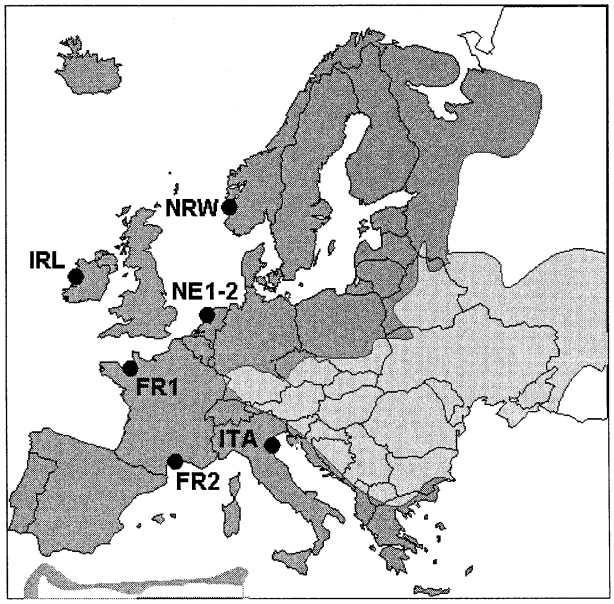

Figure 1. Sampling locations (sampling codes) and presumed natural range (darker shaded area) of European eel (*A. anguilla* L). NRW: Norway (Bergen); IRL: Ireland (Burrishoole); NE1 & NE2: the Netherlands (IJsselmeer); FR1: France (Pleurtuit); ITA: Italy (Po); FR2: France (Sète).

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 nine 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).

Data analysis of genotypes

Genetic diversity was evaluated based on genotype and allele frequencies, the level of polymorphism (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

Table 2. *Anguilla anguilla* L. Enzymes scored in tissue (liver and muscle) extracts and buffers used in the electrophoretic 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 type. Nomenclature for enzymes as in Shaklee *et al*. (1989)

Enzyme	E.C. no.	Tissue(s)	Gel buffer system (SGE + CAGE)	Locus	No. of alleles
Aspartate aminotransferase	2.6.1.1	Liver	TLCB, TM	***AAT-1****	7
				AAT-2*	3
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
				*MEP-2**	1
Malate dehydrogenase	1.1.1.37	Liver	TLCB, TM	***MDH-1****	2
				MDH-2*	5
L-lactate dehydrogenase	1.1.1.27	Muscle	P, TM	*LDH-A**	1
				*LDH-B**	1
Isocitrate dehydrogenase	1.1.1.42	Liver	TC, TM	***IDH-1****	7
				*IDH-2**	1
Fumarate hydratase	4.2.1.2	Liver	TC, TM	*FH-1**	1
				*FH-2**	1

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, 1994). In all cases significance levels were corrected for multiple comparisons using a sequential Bonferroni correction (Rice, 1989). Finally, multidimensional 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, 1994).

RESULTS

Genetic diversity

Seven of the 15 loci were polymorphic (P = 0.47). 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 (seven), with several private alleles in the former and two null alleles in the latter. 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 seven 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 ± SD) and multilocus F_{IS} estimates

Population	MNA	$P_{0.99}$	H_o	H_e (n.b.)	F_{IS}
NRW	1.7	26.7	0.057 ± 0.11	0.054 ± 0.11	-0.059^{NS}
IRL	2.1	26.7	0.049 ± 0.09	0.046 ± 0.08	-0.062^{NS}
NE1	1.8	26.7	0.048 ± 0.09	0.049 ± 0.09	0.009^{NS}
NE2	2.0	33.3	0.047 ± 0.08	0.050 ± 0.08	0.068^{NS}
FR1	1.7	33.3	0.059 ± 0.10	0.057 ± 0.10	-0.029^{NS}
FR2	1.9	33.3	0.057 ± 0.10	0.061 ± 0.11	0.074^{NS}
ITA	1.8	40.0	0.052 ± 0.09	0.049 ± 0.09	-0.061^{NS}
All populations	2.4	46.7	0.052 ± 0.09	0.052 ± 0.003	-0.001^{NS}

$^{*}P < 0.05$, ^{NS}non-significant. For population abbreviation see Table 1.

MULTILOCUS 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 replicas). 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). 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).

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 ($P > 0.1$).

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

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

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

*$P < 0.05$.

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)

	F_{ST}(θ)						
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

*$P < 0.01$, **$P < 0.001$, § = significant after Bonferroni correction.

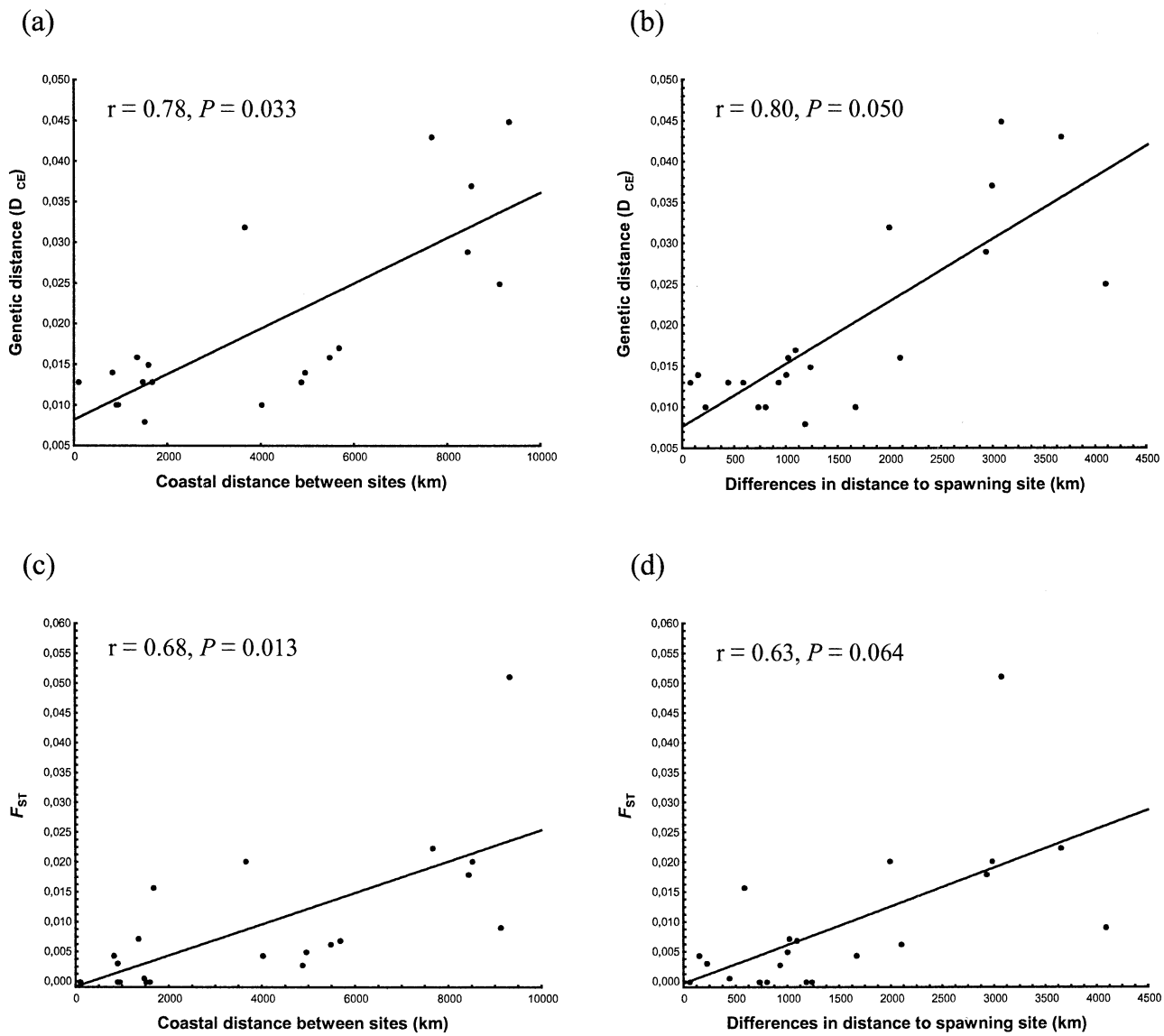

Figure 2. Test for isolation by distance in European eel. Genetic distance (D_{CE}) based on seven polymorphic allozyme loci (a) vs. coastal geographical distances and (b) vs. distance from spawning site to sampling site. Genetic differentiation (F_{ST}) based on seven polymorphic allozyme loci (c) vs. coastal geographical distances and (d) vs. distance from spawning site to sampling site. 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.

the confidence interval was considerably narrower (Fig. 3c,d).

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.

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 detect-

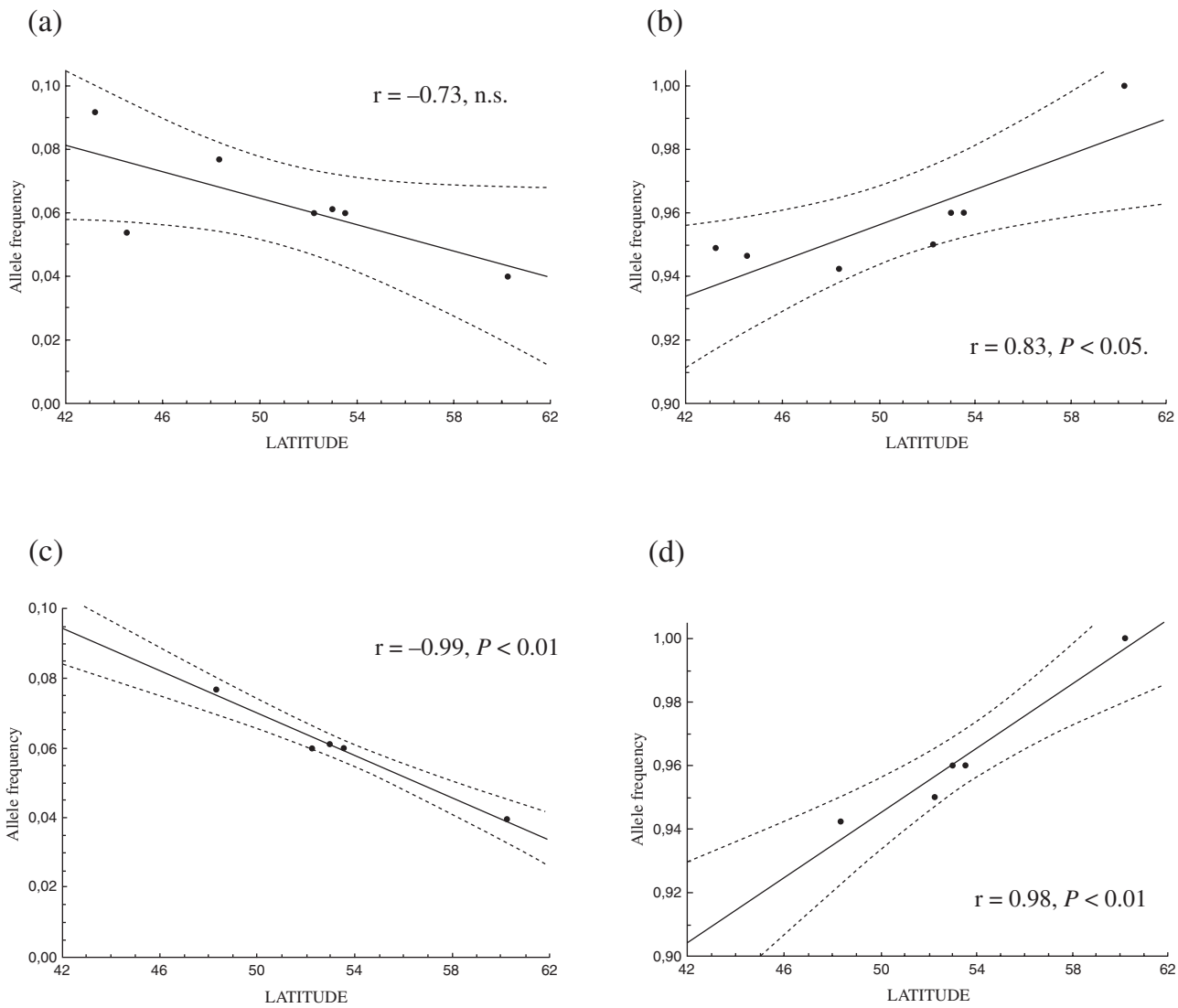

Figure 3. *Anguilla anguilla* L. Correlation between latitude (°N) and allele frequencies of (a,c) allele *GPI-1*110* and (b,d) allele *IDH-1*100*. Scatterplots (c) and (d) show the same correlation but excluding Mediterranean samples. The full line represents the regression, while the broken line indicates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

able 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 of a lower level of polymorphism, the slower evolutionary rate of coding enzymes and selective constraints (Pogson, Mesa & Boutilier, 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 ($N_e = 0.5 \times 10^6$; Daemen *et al*., 2001), the high level of enzymatic polymorphism (P = 0.47, 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

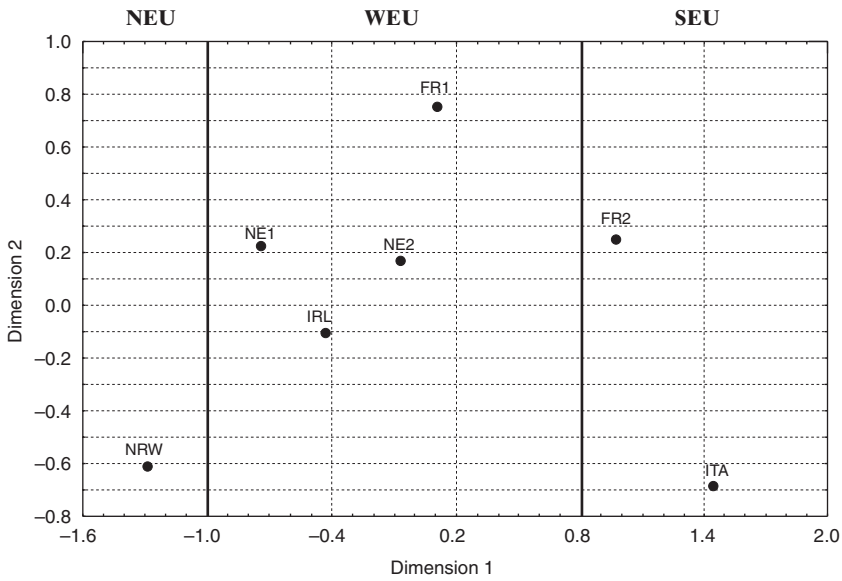

Figure 4. Multidimensional scaling plot of pairwise F_{ST}'s between seven European eel populations. Stress value = 0.002. The first axis separates all populations according to geographical distance between sites, namely Northern (NEU), Western (WEU) and Southern (SEU) Europe.

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 below), 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 (Vøllestad, 1992; McCleave *et al*., 1998). 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 Southern France (Sète) sample 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 con-

tinuous 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, 1994). 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; Daemen *et al*., 2001; Wirth & Bernatchez, 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 subpopulation 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 (5.2×10^6 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 (Grant & Bowen, 1998; Ruzzante *et al*., 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 *et al*., 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 the European eel (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; Daemen *et al*., 2001; Wirth & Bernatchez, 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 over the last two decades (Dekker, 2000).

ACKNOWLEDGEMENTS

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.

REFERENCES

Allendorf FW, Seeb LW. 2000. Concordance of genetic divergence among Sockeye Salmon populations at allozyme, nuclear DNA and mitochondrial DNA markers. *Evolution* **54:** 640–651.

Arai T, Otake T, Tsukamoto K. 2000. Timing of metamorphosis and larval segregation of the Atlantic eels *Anguilla rostrata* and *A. anguilla*, as revealed by otolith microstructure and microchemistry. *Marine Biology* **137:** 39–45.

Avise JC. 1994. *Molecular markers, natural history and evolution*. New York: Chapman & Hall.

Avise JC, Nelson WS, Arnold J, Koehn RK, Williams GC, Thorsteinsson V. 1990. The evolutionary genetic status of Icelandic eels. *Evolution* **44:** 1254–1262.

Belkhir K, Borsa P, Goudet J, Bonhomme F. 1999. *Genetix: logiciel sous Windows pour la génétique des populations*, Version 4.02. Montpellier (France): Laboratoire GENOME & POPULATION, CNRS-UPR, Université de Montpellier II.

Bernatchez L, Wilson CC. 1998. Comparative phylogeography of Nearctic and Palearctic fishes. *Molecular Ecology* **7:** 431–452.

Boëtius I, Boëtius J. 1989. Ascending elvers, *Anguilla anguilla*, from 5 European localities – Analyses of pigmentation stages, condition, chemical composition and energy reserves. *Dana. A Journal of Fisheries and Marine Research* **7:** 1–12.

Bohonak AJ. 1999. Dispersal, gene flow, and population structure. *Quarterly Review of Biology* **74:** 21–45.

Bucklin A, Wiebe PH. 1998. Low mitochondrial diversity and small effective population sizes of the copepods *Calanus finmarchicus* and *Nannocalanus minor*: possible impact of climatic variation during recent glaciation. *Journal of Heredity* **89:** 383–392.

Cavalli-Sforza LL, Edwards AWF. 1967. Phylogenetic analysis: models and estimation procedures. *Evolution* **32:** 550–570.

Chan IKK, Chan DKO, Lee SC, Tsukamoto K. 1997. Genetic variability of the Japanese eel *Anguilla Japonica* (Temminck & Schlegel) related to latitude. *Ecology of Freshwater Fish* **6:** 45–49.

Comparini A, Rodino E. 1980. Electrophoretic evidence for two species of *Anguilla* leptocephali in the Sargasso Sea. *Nature* **287:** 435–437.

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

Daemen E, Cross T, Ollevier F, Volckaert FAM. 2001. Analysis of the genetic structure of European eel (*Anguilla anguilla*) using microsatellite DNA and mtDNA markers. *Marine Biology* **139:** 755–764.

Dekker W. 2000. The fractal geometry of the European eel stock. *Ices Journal of Marine Science* **57:** 109–121.

Desaunay Y, Guérault D. 1997. Seasonal and long-term changes in biometrics of eel larvae: a possible relationship between recruitment variation and North Atlantic ecosystem productivity. *Journal of Fish Biology* **51:** 317–339.

Drilhon A, Fine JM, Amouch P, Boffa GA. 1967. Les groupes de transferrines chez *Anguilla anguilla*. Etude de deux populations d'origine géographique différente. *Comptes Rendus de l'Academie de Sciences de France* **265:** 1096–1098.

Gardner JPA, Palmer NL. 1998. Size-dependent, spatial and temporal genetic variation at a Leucine Aminopeptidase (Lap) locus among Blue Mussel (*Mytilus Galloprovincialis*)

populations along a salinity gradient. *Marine Biology* **132:** 275–281.

Grant WS, Bowen BW. 1998. Shallow population histories in deep evolutionary lineages of marine fishes: Insights from sardines and anchovies and lessons for conservation. *Journal of Heredity* **89:** 415–426.

Harding EF. 1985. On the homogeneity of the European Eel population (*Anguilla anguilla*). *Dana. A Journal of Fisheries and Marine Research* **4:** 49–66.

Harris H, Hopkinson DA. 1976. *Handbook of enzyme electrophoresis in human genetics*. Oxford: North Holland Publishing Co.

Hebert PDN, Beaton MJ. 1989. *Methodologies for allozyme analysis using cellulose acetate electrophoresis: a practical handbook*. Ontario, Canada: Helena Laboratories Inc.

Hoelzel AR. 1992. *Molecular genetic analysis of populations: a practical approach*. New York: Oxford University Press.

Horton HR, Moran LA, Ochs RS, Rawn JD, Scrimgeour KG. 1996. *Principles of biochemistry*. New Jersey: Prentice Hall Inc.

Jerry DR. 1997. Population genetic structure of the catadromus Australian Bass from throughout its range. *Journal of Fish Biology* **51:** 909–920. doi: 10.1006/jfbi.1997.0502.

Koehn RK. 1972. Genetic variation in the eel: a critique. *Marine Biology* **14:** 179–181.

Koehn RK, Williams GC. 1978. Genetic differentiation without isolation in American Eel, *Anguilla rostrata*.2. Temporal stability of geographic patterns. *Evolution* **32:** 624–637.

Kotoulas G, Bonhomme F, Borsa P. 1995. Genetic-structure of the Common Sole *Solea vulgaris* at different geographic scales. *Marine Biology* **122:** 361–375.

de Ligny W, Pantelouris EM. 1973. Origin of the European eel. *Nature* **246:** 518–519.

Lintas C, Hirano J, Archer S. 1998. Genetic variation of the European Eel (*Anguilla anguilla*). *Molecular Marine Biology and Biotechnology* **7:** 263–269.

Mantel N. 1967. The detection of disease clustering and generalised regression approach. *Cancer Research* **27:** 209–220.

Margalef R. 1985. *Western Mediterranean*. Oxford: Pergamon Press.

McCleave JD. 1993. Physical and behavioral controls on the oceanic distribution and migration of leptocephali. *Journal of Fish Biology* **43:** 243–273. doi: 10.1006/jfbi 1993.1220.

McCleave JD, Brickley PJ, O'brien KM, Kistner DA, Wong MW, Gallagher M, Watson SM. 1998. Do leptocephali of the European Eel swim to reach continental waters? Status of the Question. *Journal of the Marine Biological Association of the United Kingdom* **78:** 285–306.

McQuinn IH. 1997. Metapopulations and the Atlantic Herring. *Reviews in Fish Biology and Fisheries* **7:** 297–329.

Muss A, Robertson DR, Stepien CA, Wirtz P, Bowen BW. 2001. Phylogeography of *Ophioblennius*: the role of ocean currents and geography in reef fish evolution. *Evolution* **55:** 561–572.

Nielsen EE, Hansen MM, Schmidt C, Meldrup D, Gronkjaer P. 2001. Fisheries – Population of origin of Atlantic Cod. *Nature* **413:** 272.

Pantelouris EM, Arnason A, Tesch FW. 1970. Genetic variation in the eel. II. Transferrins, haemoglobins and esterases in the eastern North Atlantic. Possible interpretations of phenotypic frequency differences. *Genetics Research* **16:** 177–184.

Pasteur N, Pasteur G, Bonhomme F, Catalan J, Britton-Davidian J. 1987. *Manuel technique de génétique par électrophorèse des protéines*. Paris: TEC & DOC (Lavoisier).

Planes S, Doherty PJ, Bernardi G. 2001. Strong genetic divergence among populations of a marine fish with limited dispersal, *Acanthochromis Polyacanthus*, within the Great Barrier Reef and the Coral Sea. *Evolution* **55:** 2263–2273.

Planes S, Fauvelot C. 2002. Isolation by distance and vicariance drive genetic structure of a coral reef fish in the Pacific Ocean. *Evolution* **56:** 378–399.

Pogson GH, Mesa KA, Boutilier RG. 1995. Genetic population structure and gene flow in the Atlantic Cod *Gadus morhua* – a comparison of allozyme and nuclear RFLP loci. *Genetics* **139:** 375–385.

Powers DA, Lauerman T, Crawford D, Dimichele L. 1991. Genetic mechanisms for adapting to a changing environment. *Annual Review of Genetics* **25:** 629–659.

Raufaste N, Bonhomme F. 2000. Properties of bias and variance of two multi-allelic estimators of F_{ST}. *Theoretical Population Biology* **57:** 285–296. doi: 10.1006_tpbi.2000.1457.

Raymond M, Rousset F. 1995. *GENEPOP* Version 1.2.: a population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86,** 248–249.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* **43:** 223–225.

Richardson BJ, Baverstock PR, Adams M. 1986. *Allozyme electrophoresis: a handbook for animal systematics and population studies*. San Diego: Academic Press Inc.

Robertson A, Hill WG. 1984. Deviations from Hardy–Weinberg proportions – Sampling variances and use in estimation of inbreeding coefficients. *Genetics* **107:** 703–718.

Ruzzante DE, Taggart CT, Cook D. 1998. A nuclear DNA basis for shelf- and bank-scale population structure in Northwest Atlantic Cod (*Gadus morhua*): Labrador to Georges Bank. *Molecular Ecology* **7:** 1663–1680.

Shaklee JB, Allendorf FW, Morizot DC, Whitt GS. 1989. Standardized nomenclature for protein-coding loci in fish. *Fisheries* **14:** 2–3.

Shaw PW, Pierce GJ, Boyle PR. 1999. Subtle population structuring within a highly vagile marine invertebrate, the veined Squid *Loligo forbesi*, Demonstrated with microsatellite DNA markers. *Molecular Ecology* **8:** 407–417.

Sinclair M. 1988. *Marine populations. an assay on population regulation and speciation*. Seattle: University of Washington Press.

Sokolova IM, Portner HO. 2001. Temperature effects on key metabolic enzymes in *Littorina saxatilis* and *L. obtusata* from different latitudes and shore levels. *Marine Biology* **139:** 113–126.

Statsoft Inc. 1994. *STATISTICA: For windows operating system*. Tulsa, OK: Statsoft Inc.

Stepien CA. 1999. Phylogeographical structure of the Dover Sole *Microstomus pacificus*: the larval retention hypothesis

and genetic divergence along the deep continental slope of the North-eastern Pacific Ocean. *Molecular Ecology* **8:** 923–939.

Tesch FW. 1977. *The eel, biology and management of anguillid eels*. London: Chapman & Hall.

't Land JV, Van Putten WF, Villarroel H, Kamping A, Van Delden W. 2000. Latitudinal variation for two enzyme loci and an inversion polymorphism in *Drosophila melanogaster* from central and South America. *Evolution* **54:** 201–209.

Tsukamoto K, Nakai I, Tesch WV. 1998. Do all freshwater eels migrate? *Nature* **396:** 635–636.

Vøllestad LA. 1992. Geographic-Variation in Age and Length at Metamorphosis of Maturing European Eel – Environmental-Effects and Phenotypic Plasticity. *Journal of Animal Ecology* **61:** 41–48.

Waples RS. 1987. A multispecies approach to the analysis of gene flow in marine shore fishes. *Evolution* **41:** 385–400.

Waples RS. 1998. Separating the wheat from the chaff: Patterns of genetic differentiation in high gene flow species. *Journal of Heredity* **89:** 438–450.

Ward RD, Woodwark M, Skibinski DOF. 1994. A comparison of genetic diversity levels in marine, freshwater and anadromous fishes. *Journal of Fish Biology* **44:** 213–232. doi: 10.1006/jfbi 1994.1021.

Whitmore DH. 1990. *Electrophoretic and isoelectric focusing techniques in fisheries management*. Boston: CRC Press.

Williams GC, Koehn RK, Mitton JB. 1973. Genetic differentiation without isolation in American Eel, *Anguilla rostrata*. *Evolution* **27:** 192–204.

Wirth T, Bernatchez L. 2001. Genetic evidence against panmixia in the European Eel. *Nature* **409:** 1037–1040.

Yahyaoui A, Bruslé J, Pasteur N. 1983. Etude du polymorphisme biochimique de deux populations naturelles (Maroc Atlantique et Rousillon) de civelles et anguillettes d'*Anguilla anguilla* L. et de deux échantillons d'élevages. *IFREMER Actes de Colloques* **1:** 373–390.

APPENDIX

Allele frequencies at seven polymorphic allozyme loci in seven 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	0.0000	0.0000	0.0100	0.0000	0.0000	0.0000	0.0000	0.0014
75	0.0000	0.0000	0.0000	0.0200	0.0192	0.0204	0.0179	0.0111
80	0.0100	0.0000	0.0100	0.0000	0.0000	0.0000	0.0357	0.0080
90	0.0000	0.0000	0.0100	0.0000	0.0000	0.0000	0.0000	0.0014
100	0.9600	1.0000	0.9600	0.9500	0.9423	0.9490	0.9464	0.9582
110	0.0300	0.0000	0.0100	0.0200	0.0385	0.0204	0.0000	0.0170
120	0.0000	0.0000	0.0000	0.0100	0.0000	0.0102	0.0000	0.0029
H_e	0.0774	0.0000	0.0780	0.0966	0.1102	0.0985	0.1027	0.0772
H_o	0.0800	0.0000	0.0800	0.1000	0.1154	0.1020	0.1071	0.0792
*GPI-1**								
(*N*)	49	50	50	50	26	49	28	302
80	0.0102	0.0000	0.0200	0.0000	0.0000	0.0102	0.0714	0.0160
90	0.0612	0.1300	0.0400	0.0800	0.0769	0.1020	0.0000	0.0700
100	0.8673	0.8300	0.8800	0.8600	0.8462	0.7959	0.8750	0.8506
110	0.0612	0.0400	0.0600	0.0600	0.0769	0.0918	0.0536	0.0634
H_e	0.2401	0.2926	0.2200	0.2504	0.2722	0.3476	0.2264	0.2689
H_o	0.2245	0.3000	0.2400	0.2400	0.2692	0.3469	0.2500	0.2682
*AAT-1**								
(*N*)	50	50	50	50	26	49	28	303
33	0.0000	0.0200	0.0100	0.0100	0.0192	0.0102	0.0000	0.0099
60	0.0200	0.0000	0.0300	0.0000	0.0385	0.0306	0.0357	0.0221
80	0.0500	0.0500	0.0300	0.0200	0.0385	0.0612	0.0536	0.0433
90	0.0000	0.0200	0.0100	0.0200	0.0000	0.0000	0.0179	0.0097
100	0.9100	0.9000	0.9000	0.9300	0.9038	0.8776	0.8571	0.8969
110	0.0100	0.0100	0.0200	0.0100	0.0000	0.0204	0.0357	0.0152
133	0.0100	0.0000	0.0000	0.0100	0.0000	0.0000	0.0000	0.0029
H_e	0.1688	0.1866	0.1876	0.1340	0.1797	0.2247	0.2596	0.1885
H_o	0.1600	0.2000	0.2000	0.1400	0.1923	0.1837	0.2857	0.1881

Appendix *Continued*

	NE1	NRW	IRL	NE2	FR1	FR2	ITA	Total
*AAT-2**								
(*N*)	50	50	50	50	26	50	28	304
90	0.0000	0.0000	0.0100	0.0000	0.0000	0.0000	0.0000	0.0014
100	1.0000	0.9800	0.9900	0.9900	0.9808	0.9800	1.0000	0.9887
110	0.0000	0.0200	0.0000	0.0100	0.0192	0.0200	0.0000	0.0099
H_e	0.0000	0.0392	0.0198	0.0198	0.0377	0.0392	0.0000	0.0228
H_o	0.0000	0.0400	0.0200	0.0200	0.0385	0.0400	0.0000	0.0230
*MDH-1**								
(*N*)	50	50	50	50	26	50	28	304
100	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9821	0.9974
110	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0179	0.0026
H_e	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0351	0.0033
H_o	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0357	0.0033
*MDH-2**								
(N)	50	50	50	50	26	50	28	304
70	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0179	0.0026
80	0.0400	0.0500	0.0700	0.0600	0.0769	0.0400	0.0000	0.0481
90	0.0400	0.0500	0.0100	0.0200	0.0192	0.0700	0.0179	0.0324
100	0.8700	0.8400	0.9000	0.8900	0.8654	0.8900	0.9643	0.8885
110	0.0500	0.0600	0.0200	0.0300	0.0385	0.0000	0.0000	0.0284
H_e	0.2374	0.2858	0.1846	0.2030	0.2433	0.2014	0.0695	0.2125
H_o	0.2600	0.3200	0.2000	0.1600	0.2692	0.1800	0.0714	0.2138
*PGM-1**								
(*N*)	50	50	50	50	26	50	28	304
90	0.0000	0.0000	0.0000	0.0200	0.0000	0.0000	0.0179	0.0054
100	1.0000	1.0000	1.0000	0.9800	1.0000	1.0000	0.9821	0.9946
H_e	0.0000	0.0000	0.0000	0.0392	0.0000	0.0000	0.0351	0.0098
H_o	0.0000	0.0000	0.0000	0.0400	0.0000	0.0000	0.0357	0.0099