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Genetic structure of the flounders *Platichthys flesus* and *P. stellatus* at different geographic scales

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Abstract The genetic structure of the flounders *Platichthys flesus* L. and *P. stellatus* Pallas was investigated on different spatial scales through analysis of allozyme variation at 7 to 24 polymorphic loci in samples collected from different regions (Baltic Sea, North Sea, Brittany, Portugal, western Mediterranean, Adriatic Sea, Aegean Sea and Japan) in 1984 to 1987. No geographic variation was evident within a region. Some pattern of differentiation by distance was inferred within the Atlantic, while the Mediterranean comprised three geographically isolated populations and was itself geographically isolated from the Atlantic (fixed allele differences at up to three loci were found among *P. flesus* populations from the Atlantic, the western Mediterranean, the Adriatic Sea, the Aegean Sea and also *P. stellatus* from the coast of Japan). Sea temperature during the reproductive period probably acts as a barrier to gene flow between populations. Genetic distances among European flounder populations (*P. flesus*) were higher than, or of the same magnitude as, the genetic distance between Pacific (*P. stellatus*) and European flounder populations, suggesting that *P. flesus* is paraphyletic and/or there is no phylogenetic basis to recognising *P. stellatus* as a different species. The divergence between *P. flesus* and *P. stellatus* was thus inferred to be more recent than the divergence between the present *P. flesus* populations from the NE Atlantic and eastern Mediterranean. The eastern Mediterranean populations are thought to originate from the colonisation of the Mediterranean by a proto-*P. flesus*/*P. stellatus* ancestor, whereas the present western Mediterranean population has undergone a more recent colonisation event by *P. flesus*. Patterns of mitochondrial DNA variation, established on a smaller array of *P. flesus* samples, were in accordance with the geographic patterns inferred from the allozyme survey. In addition, they supported the hypothesis of a two-step colonisation of the western Mediterranean. These results contribute to our understanding of the biogeography of the Mediterranean marine fauna, especially the group of boreal remnants to which *P. flesus* belongs.

Introduction

According to the present geographic distribution of species, faunal units have been recognised in the northeastern (NE) Atlantic/Mediterranean Sea region and referred to as Tethys relicts, Black Sea relicts, boreal immigrants, tropical immigrants and Mediterranean endemics (Tortonese 1964; Klausewitz 1973; Quignard 1978). About one-fifth of the Mediterranean fish species and subspecies are endemic, a proportion considered to be sufficient for recognising the Mediterranean Sea as a biogeographic province on its own [Quignard 1978; but see Ekman (1968) for a different interpretation]. Nearly four-fifths of the Mediterranean fish species also occur in the Atlantic and are thought to have invaded the Mediterranean since the opening of the Gibraltar Strait in the early Pliocene (Briggs 1974; Quignard 1978). Morphological divergence based on, for example, the number of fin rays or vertebrae in several fish species (e.g. the hake *Merluccius merluccius*, the pilchard *Sardina pilchardus* and the Spanish sardine *Sardinella aurita*) has been thought to reflect a steady state of equilibrium in the present exchange of fish fauna through the Gibraltar Strait (Quignard 1978). Within the Mediterranean, it is not clear whether the western and eastern basins should be considered as distinct biogeographic entities, nor whether the Adriatic Sea and Aegean Sea faunas have undergone separate evolution (Tortonese 1971; Econo-

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midis and Bauchot 1976; Quignard 1978). Furthermore, there is some disagreement as to whether latitudinal clines in the Mediterranean Sea should be recognised or not (Quignard 1978).

Our concern here is to refine our understanding of the history and evolution of the NE Atlantic/Mediterranean marine fauna and of the biogeographic relationships between oceanographical sub-basins (i.e. NE Atlantic, western Mediterranean, Adriatic Sea, Aegean Sea, Black Sea). For this, we addressed the infra-specific level of geographic structure using genetic markers, as a detailed picture of the contemporary population genetic architecture is expected to reveal patterns of gene flow, the occurrence of geographic barriers, and relative times of divergence elapsed since the geographic separation of populations.

The flounder *Platichthys flesus*, one of the few boreal immigrants still present in the Mediterranean Sea, is an appropriate species with which to investigate the evolutionary history of the fauna of the NE Atlantic/Mediterranean Sea. It ranges from the White Sea to southern Portugal; and in the western Mediterranean, the Adriatic Sea, the Aegean Sea at latitudes above 40°N and the Marmara/Black/Azov Seas (maps of distribution in Berrebi et al. 1985 and Borsa 1986, and references therein), where it is common in estuaries, lagoons and shallow seas. These habitats are distributed in a discontinuous fashion along the European coastline. On the one hand, such a geography limits the movements of adults and may reduce genetic exchange among populations, but on the other hand the dispersal potential of the pelagic eggs of the flounder (development time = 7 to 5 d at 4 to 11.5 °C: Ehrenbaum 1909) and its larvae (at least 12 d in plankton after hatching, at 12 °C: Specchi et al. 1979) may appear sufficient to allow effective connection. How much the populations from different localities exchange genes can be inferred from the patterns of genetic differentiation.

Two endemic subspecies of *Platichthys flesus*, *P. flesus italicus* and *P. flesus luscus* have been described in, respectively, the Adriatic Sea and the Black Sea on morphological and genetical grounds (Norman 1934; Bini 1968; Galleguillos and Ward 1982). Another species, the starry flounder *P. stellatus*, is similar to *P. flesus* in both its morphology and its ecology (Shmidt 1965); its range extends from the Chukchi Sea to the coast of Honshu and to the coast of California. We conducted allozyme analyses on a range of samples of *P. flesus* from the NE Atlantic to the eastern Mediterranean and *P. stellatus* from Honshu. Different geographic scales were addressed in the analysis of allozyme variation: within-subpopulation; between sub-populations within an oceanographic sub-basin or "region"; between regions within a basin (i.e. NE Atlantic and Mediterranean/Black Sea). Relating the degree of genetic differentiation to spatial scale makes it possible to define the limit beneath which populations can be considered panmictic or nearly so, and above which they can be considered genetically structured. The analysis also included the results of an earlier survey of genetic variation in *P. flesus* (Galleguillos and Ward 1982) based on partially different samples of populations and loci. Complementary information was provided by mitochondrial (mt) DNA variation in a set of *P. flesus* samples from a more restricted range.

Materials and methods

Collection and processing of samples

Samples of *Platichthys flesus* L. and *P. stellatus* Pallas were collected by bottom trawling in various locations in the Baltic Sea, the North Sea, the coast of Brittany, the coast of Portugal, the Mediterranean Sea (Fig. 1; Table 1) and the Pacific Ocean coast of Japan (Table 1) between 1984 and 1987. The samples from lagoons in Golfe du Lion (western Mediterranean Sea) and the Adriatic Sea were obtained using the networks of nets and traps ("capetchades") set up by local fishermen.

The morphological and meristic characters of the individuals from the Atlantic conformed to the *Platichthys flesus flesus* subspecies type (Berg 1932; type collected in the North Sea), and those from the Adriatic Sea, to *P. flesus italicus* (Galleguillos and Ward 1982). The flounder from the Aegean Sea is close to *P. flesus italicus* according to meristic data, and close to *P. flesus flesus* from the Atlantic according to morphology (Vianet 1985), but is considered to be *P. flesus luscus* by Economidis and Bauchot (1976). The morphology of the flounder from Golfe du Lion corresponds to that of *P. flesus flesus*, although statistically significant differences have been reported (Vianet 1985). The sample from Japan was assigned to the species *P. stellatus* according to Norman (1934).

Immediately after collection, ~1 cm^3 of each liver and muscle tissues was dissected from each individual and homogenised at 0 to 4 °C in an equal volume of 0.1 *M* Tris/HCl/EDTA/NADP buffer (pH 6.8). The supernatant obtained after 30 min centrifugation at 22 000 ×*g* at 4 °C was used as the soluble enzyme extract. It was stored at −70 °C until it was run on horizontal 12% starch gels for isozyme electrophoresis according to protocols detailed in Pasteur et al. (1987). The staining procedures were those of Pasteur et al. for all enzymes except creatine kinase (CK). We identified CK as the most abundant soluble protein in flounder muscle-tissues, hence instead of the costly, specific-CK histochemical stain we used a general-protein stain (Pasteur et al. 1987). Different esterase (EST) functions were revealed using the combinations of specific enzyme substrates and inhibitors reported in Berrebi et al. (1990) (see also Pasteur et al. 1987, their Fig. 2–21, p. 44).

The Mendelian determination of some of the enzyme polymorphisms, at loci encoding the muscle glycerophosphate dehydrogenase, glucose-phosphate isomerases (GPI) GPI-1 and GPI-2, the malate dehydrogenase MDH-2 and phosphoglucomutase, has been confirmed in the plaice *Pleuronectes platessa* (Purdom et al. 1976; Ward and Beardmore 1977), another flatfish species genetically close to *Platichthys flesus* (Ward and Galleguillos 1983). Electrophoretic variation observed for all enzymes in the present study (Table 2) except CK and the glucose-phosphate isomerase GPI-2 conformed to a simple Mendelian model of co-dominance and to the known quaternary structure of each enzyme in vertebrates (Harris and Hopkinson 1976; Ward and Beardmore 1977; Richardson et al. 1986). Patterns observed for CK had two instead of the three bands expected of a dimeric enzyme in presumed heterozygotes, a phenomenon that is typical for teleosts and possibly due to temporal or spatial isolation of the synthesis and assembly of the allelic enzyme subunits within the cell (Ferris and Whitt 1978). GPI zymograms consist of three zones of migration (Pasteur et al. 1987, their Fig. 2–9, p. 29), but in some individuals the expected fastest isozyme was not present, a phenomenon that we interpreted as the expression of a "null" allele encoding an inactive form of the enzyme. Presumed homozygotes for the inactive electromorph at Locus *GPI-2* were scored at substantial frequencies

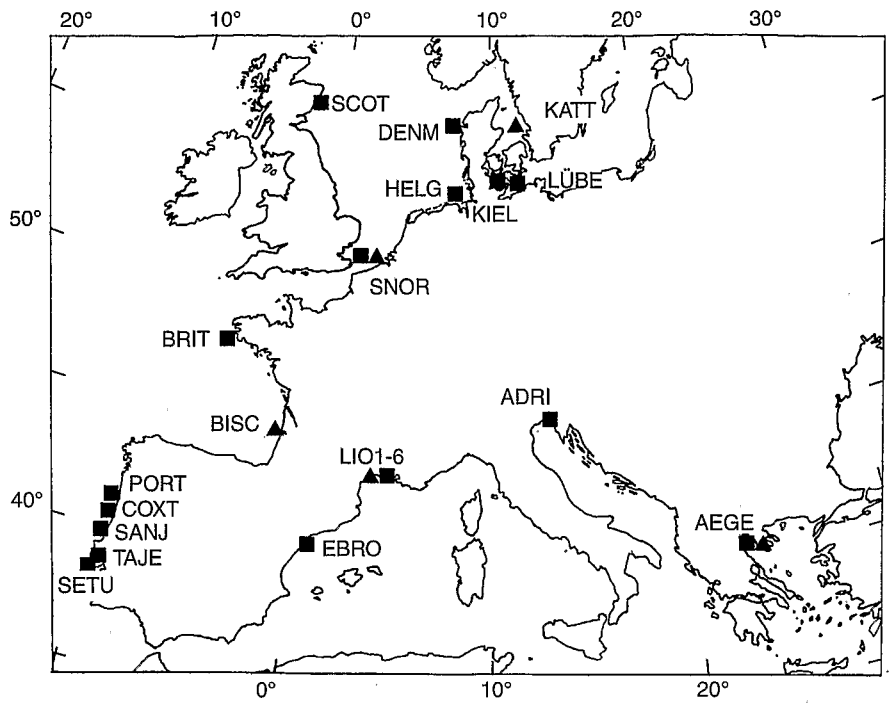

Fig. 1 Sampling locations for *Platichthys flesus* (■ samples subjected to allozyme analysis; ▲ samples subjected to mitochondrial DNA analysis; *KATT* Kattegat; *BISC* Biscaye Gulf; *other abbreviations* as in Table 1)

Table 1 *Platichthys flesus* and *P. stellatus*. Details of sampling locations and dates, sample size (*N*) and number of loci analysed (*r*)

Region, locality	Date	(*N*)	*r*
Baltic Sea			
LÜBE (Lübeck bay)	Apr. 1986	(25)	8
KIEL (Kiel bay)	Apr. 1986	(37)	8
North Sea			
DENM (West coast of Denmark)	Feb. 1987	(9)	8
HELG (Helgoland bay)	Feb. 1987	(34)	8
SCOT (Aberdeen)	Feb. 1987	(33)	8
SNOR (Southern North Sea, between Thames estuary and Rhine estuary)	Feb. 1987	(54)	8
Brittany			
BRIT (South of Brittany peninsula)	Feb. 1987	(10)	8
Portugal			
PORT (Porto)	Sep. 1987	(15)	7
COXT (Coxta Nova, Aveiro)	Feb. 1987	(47)	7
SANJ (San Jacinto)	May 1987	(23)	7
SETU (Setubal, south of Lisboa)	Feb. 1987	(25)	7
TAJE (Taje estuary)	Sep. 1984	(35)	23
Western Mediterranean			
EBRO (Ebro delta, Spain)	Feb. 1985	(5)	13
LIO1 (Agde, Golfe du Lion)	Feb.–Mar. 1985	(19)	7
LIO2 (Ingril lagoon, Golfe du Lion)	Nov.–Dec. 1985	(24)	16
LIO3 (La Peyrade lagoon, Golfe du Lion)	Apr.–May 1984	(133)	6
LIO4 (Mauguio lagoon, Golfe du Lion)	Nov.–Dec. 1984	(33)	7
LIO5 (Grau du Roi, Golfe du Lion)	Feb. 1985	(167)	7
LIO6 (Saintes-Maries de la Mer, Golfe du Lion)	Feb.–Mar. 1986	(26)	33
Adriatic Sea			
ADRI (Marano lagoon, gulf of Trieste)	Mar. 1986	(22)	33
Aegean Sea			
AEGE (Thermaikos gulf)	Apr. 1986	(20)	33
Japan			
JAPN (Tokyo bay, Honshu: *P. stellatus*)	Feb.–June 1986	(22)	22

(~0.05) in both the Golfe du Lion and Adriatic Sea samples. Although no activity was detected, it was possible to infer the mobility of the null electromorphs from the mobility of the hybrid GPI molecule migrating at a position intermediate between the products of *GPI-1* and *GPI-2* (see Fig. 2–9, p.29 of Pasteur et al. 1987). The mobilities of the null electromorphs were identical to those of electromorphs GPI-2^{100} and GPI-2^{102} in the Golfe du Lion samples and the Adriatic Sea sample, respectively. It was not possible to directly estimate the frequencies of such null electromorphs, as heterozygous individuals cannot be identified if the null allele encodes a null electromorph of the same mobility as the active electromorph. Hence these were pooled within one of two electrophoretic classes, namely GPI-2^{100} and GPI-2^{102}, in Golfe du Lion and the Adriatic Sea, respectively.

Analysis of enzyme polymorphism

Genotypic disequilibria (Weir 1990) were calculated from genotype data for pairs of loci using the LINKDIS procedure implemented in the package GENETIX (Belkhir et al. 1996), and were tested against the null hypothesis of independence of loci in each subpopulation using the chi-square test following Black and Krafsur (1985): $\chi^2 = N \sum_i \sum_{i'} (\Delta_{ii'}{}^2 : x_i x_{i'})$, with $(m-1)\cdot(n-1)$ degrees of freedom, where N = sample size; $\Delta_{ii'}$ = value of genotypic disequilibrium calculated on allele i at the first locus and allele i' at the second locus; x_i and $x_{i'}$ = frequencies of i and i'; and m and n = numbers of alleles at the two loci.

Deviations from Hardy–Weinberg genotypic proportions were estimated in each population at the regional scale by calculating the across-loci unweighted average of Weir and Cockerham's (1984) f-statistic, the estimator of the fixation index within subpopulations. The f-estimates were calculated on either single samples (Brittany, the Adriatic Sea, the Aegean Sea and Japan) or as weighted averages (see Weir and Cockerham 1984) on sets of several samples considered as replicates (those of each the Baltic Sea, the North Sea, Portugal and the western Mediterranean).

G_{ST}, Nei's (1973) parameter for the apportion of genetic diversity within and across subpopulations (k = 1 to s) was estimated over polymorphic loci as $G_{ST} = 1 - (1{:}s \cdot \sum_k H_k){:}H_T$, where H_k = average gene diversity across loci (j = 1 to r) in subpopulation $k (H_k = 1{:}r \cdot \sum_j h_{jk})$ and H_T = average gene diversity across loci in the total population $(H_T = 1{:}r \cdot \sum_j h_{Tj})$. The gene diversities at a locus were estimated from the electromorph frequencies (x_{ijk}) as: $h_{jk} = 2N_k{:}\,(2N_k - 1)\cdot(1 - \sum_i x_{ijk^2})$ and $h_{Tj} = 2\sum_k N_k{:}\,(2\sum_k N_k - 1)\cdot(1 - 1{:}\sum_k N_k \cdot \sum_i \sum_k N_k x_{ijk^2})$, where N_k = sample size.

We considered that the relative variation in gene diversity at a locus is replicated across loci over the whole genome, hence internal resampling using the jackknife procedure (Sokal and Rohlf 1981) was made across loci for estimating the variance of G_{ST}. The jackknife was carried out by dropping each line (gene diversities at a locus) in turn from the data and analysing the remaining data. The inferred pseudodistribution of G_{ST} was then compared to a normal distribution according to Lilliefors (1967) using the test for normality implemented in the package BIOMECO (Lebreton et al. 1990). Normality was rejected if the probability of the largest difference was <0.20 after Bonferroni-type correction for multiple tests (Weir 1990). Otherwise, we assumed that the jackknife pseudovalues of G_{ST} were normally distributed, and the null hypothesis of $G_{ST} = 0$ was tested by a one-tailed Student's t-test with $r - 1$ degrees of freedom. Note that the subpopulation component of average gene diversity was unweighted by sample size. For small G_{ST} values, this practice led to artifactual, negative estimates, but on the other hand this equal weighting has been advocated for large G_{ST} (Chakraborty and Leimar 1987).

The analysis addressed the within-"region" scale (different subpopulations separated by ~100 to 800 km within a group) and the within-basin scale (different regions in an oceanographic basin, namely the Atlantic and the Mediterranean Sea), and was extended to include both data from the present survey and the electromorph-frequency data presented in Galleguillos and Ward (1982; their appendix). Three sampling areas, namely Kiel bay (Sample DEN of Galleguillos and Ward), the southern North Sea (their sample LON) and the Adriatic Sea (their samples ADRI and ADRII) and 23 loci (see Tables 3 to 5) were common to both our study and that of Galleguillos and Ward. Areas sampled exclusively by Galleguillos and Ward were Wales, South-West England (both hereafter grouped into the "South-West (SW) Britain" region), the Marmara Sea and the Black Sea. For comparisons involving the data from both surveys, identities of the most common electromorphs at a locus (see Tables 3 to 5) were first assumed from the observation that their frequencies in the above common locations were identical or similar between the two studies. This was then confirmed by checking the relative mobilities of the other electromorphs, and further by using a control made up of 10–18 individuals of the plaice *Pleuronectes platessa* sampled in the North Sea, whose electromorph mobilities were used as the reference by Galleguillos and Ward, following Ward and Beardmore's (1977) earlier study. It was usually not possible to ascertain the identities of rarer electromorphs. Hence these were pooled within two other, synthetic electromorph classes (i.e. faster and slower) and the gene diversities were subsequently re-calculated. We were unable to draw a clear correspondence between Galleguillos and Ward's aspartate aminotransferase locus *Got-2* and EST locus *Est-4* (their notation) and the present study's *AAT-L2* or *AAT-3* and *EST-L2*, *EST-L3* or *EST-4*, respectively (see Table 2 for full locus names). None of these loci, then, was used for comparisons involving data from both studies.

Genetic-distance (D; Nei 1972) calculations were made on all possible pairs of populations shown to be geographically isolated from each other [i.e. Atlantic (Sample TAJE), Golfe du Lion, Adriatic Sea, Aegean Sea and Japan]. The sample of loci on which the calculations of D were based included all loci in common to the pair of populations under comparison. Different loci have different levels of polymorphism and presumably undergo different rates of evolution. Ideally, either a large number or the same set of loci should be scored in all populations to avoid undesirable bias in the comparison of D values. Here the loci scored were not the same across populations (Table 1), as the number of shared loci varied according to the pair of samples under scrutiny, but a reasonable number of loci (≥16) was shared in all populations out of a maximum of 32 scored in the present study (*EST-L3* was not taken into account because of its linkage with *EST-4* (see "Results")). Assuming a positive relationship between genetic-distance and evolutionary time, the comparison of pairwise genetic-distance values adds a historical dimension to a species' genetic structure and infraspecific evolutionary processes. Galleguillos and Ward (1982) made the assumption of a linear relationship whereby 1 unit of D is equivalent to 19 million years following Vawter et al. (1980), an equivalence that we keep here for comparative purposes. However, comparing the ranks of D values is sufficient for proposing an evolutionary scenario based on the sequence of events, and does not require the assumption of linearity. Dendrograms were constructed using the unweighted pair-grouping algorithm (UPGMA; Sokal and Michener *in* Nei 1987) on the matrix of pairwise D for illustrating the hypothetical phylogenetic relationships between geographically isolated populations.

The null hypothesis of no correlation of genetic distance (D) with geographic distance (d) was tested using Mantel's non-parametric test on pairwise distance matrices (Manly 1985) using the MANTEL procedure in GENETIX (Belkhir et al. 1996). Two genetic distances were used according to the level of population structure: Nei's D between geographically isolated populations, and a log-transform of $1\text{-}G_{ST}$ (Reynolds et al. 1983) between sub-populations in the Atlantic. The choice of distance parameter depended on the scale of differentiation: Nei's D is more appropriate for comparing geographically isolated populations which have accumulated genetic differences through both the processes of drift and mutation, while the distance parameter of Reynolds et al. has been designed to measure divergence between populations due only to drift (no mutation is assumed here). d between the southern North Sea and Japan was measured along the coastline on a 1:160 million north-polar azimuthal equidistant projection map of the world (Anonymous 1989); d betweeen locations in Europe was measured simi-

Table 2 *Platichthys flesus* and *P. stellatus*. Enzymes scored in tissue (liver and muscle) extracts, and buffers used in the electrophoretic analysis [*PC* phosphate-citrate buffer, pH 6.3 (Pasteur et al. 1987); Poulik discontinuous tris-citrate buffer, pH 8.7 (Poulik 1957); *TC* tris-citrate buffer, pH 8.0 (Selander et al. 1971); *TCBL* discontinuous Tris-citrate-borate-LiOH buffer, pH 7.0 (Pasteur et al. 1987); *TG* tris-glycine buffer, pH 9.0 (Pasteur et al. 1987); *TME* tris-maleate-EDTA buffer, pH 6.9 (Pasteur et al. 1987); *nomenclature for enzymes and loci* as in Shaklee et al. (1990)]. Loci in parentheses are homologous loci in the plaice *Pleuronectes platessa* (Ward and Beardmore 1977)

Enzyme (abbreviation; E.C. No.)	No. of loci		Buffer	Presumed loci
	liver	muscle		
Adenosine deaminase (ADA; 3.5.4.4)		1	TME	*ADA* (*Ada*)
Adenylate kinase (AK; 2.7.4.3)		1	PC	*AK*(*Ak*)
Alcohol dehydrogenase (ADH; 1.1.1.1)	1		TC	*ADH*
Alkaline phosphatase (AKP; 3.1.3.1)		1	TG	*AKP*
Aspartate aminotransferase (AAT; 2.6.1.1)	2	1	PC, TG	*AAT-L1* (*Got-1*), *AAT-L2*, *AAT-3*
Creatine kinase (CK; 2.7.3.2)		1	TCBL	*CK* (*Ck-M*)
Naphthyl-esterase (EST; 3.1.1.-)	3	1	TCBL	*EST-L1* (*Est-2*), *EST-L2*, *EST-L3*, *EST-4*
4-methyl-umbelliferyl-esterase (EST-D; 3.1.1. -)	1		TCBL	*EST-D* (*Est-D*)
Fumarate hydratase (FUM; 4.2.1.2)		1	Poulik	*FUM*
Glucose-phosphate isomerase (GPI; 5.3.1.9)		2	Poulik	*GPI-1* (*Pgi-1*), *GPI-2* (*Pgi-2*)
Glycerophosphate dehydrogenase (GPDH; 1.2.1.12)	1	1	TC	*αGPDH-L* (*αGpdh-2*), *αGPDH-M*(*αGpdh-1*)
Glyoxalase (GLO; 4.4.1.5)		1	TC	*GLO* (*Glo-1*)
Isocitrate dehydrogenase (IDH; 1.1.1.42)	1	1	TC	*IDH-L* (*Idh-1*), *IDH-M* (*Idh-2*)
Lactate dehydrogenase (LDH; 1.1.1.27)		1	Poulik	*LDH*(*Ldh-1*)
Malate dehydrogenase (MDH, 1.1.1.37)	1	2	PC	*MDH-1, MDH-M1* (*Mdh-1*), *MDH-M2*(*Mdh-2*)
Malic enzyme (ME; 1.1.1.40)		1	TCBL	*ME-2* (*Me*)
Mannose-phosphate isomerase (MPI; 5.3.1.8)		1	TCBL	*MPI*
Phosphoglucomutase (PGM; 2.7.5.1)		1	Poulik	*PGM* (*Pgm-1*)
Phosphogluconate dehydrogenase (6PGDH; 1.1.1.44)	1		PC	*6PGDH* (*6Pgdh*)
Octanol dehydrogenase (ODH; 1.1.1.-)	1		TC	*ODH* (*Odh*)
Sorbitol dehydrogenase (SDH; 1.1.1.14)	1		TG	*SDH* (*Sdh*)
Superoxide dismutase (SOD; 1.15.1.1)	1		Poulik	*SOD* (*Sod*)
Xanthine dehydrogenase (XDH; 1.2.1.37)	1		Poulik	*XDH* (*Xdh*)

larly, on a 1:2 350 000 polyconic projection map of Europe (Anonymous 1989).

Mitochondrial DNA procedures and data analysis

Restriction fragment-length patterns (RFLP) were obtained on the total mtDNA of 21 individuals collected in 1987: four from the entrance of the Baltic Sea (Sample KATT on Fig. 1), three from the North Sea (SNOR), three from the Biscaye Gulf (BISC), eight from Golfe du Lion (LION) and three from the Aegean Sea (AEGE).

The liver and spleen of each individual were dissected from the live fish and preserved in liquid nitrogen until homogenisation in a 10 m*M* NacCl, 10 m*M* Tris, 1 m*M* EDTA, pH 7.8 solution using a ratio of 5 ml solution per 1 g tissue. The mitochondria were isolated by two cycles of centrifugation and the mtDNA was phenol-chloroform extracted according to Boursot et al. (1987). It was then digested in separate reactions by restriction enzymes *Ava II* and *Hpa II* (Boehringer, Mannheim) according to the manufacturer's instructions. The reaction mixture was subjected to electrophoresis on vertical 5% polyacrylamide gels and the mtDNA restriction fragments were visualised using silver-staining according to Guillemette and Lewis (1983).

The presence or absence in an individual of a mtDNA restriction fragment of a given size was considered as a single character with State 1 or 0, respectively. The matrix of individuals × characters was subjected to parsimony analysis and an unrooted phylogenetic tree was inferred, using the MIX procedure of the PHYLIP package (Felsenstein 1989). The robustness of branchings at each node was empirically tested by 50 bootstrap resamplings of the original data matrix (Felsenstein 1985) using the bootstrapping procedure implemented in PHYLIP.

Results

Electromorph frequencies at up to 33 presumptive allozyme loci in populations of *Platichthys flesus* and *P. stellatus* are shown in Tables 3 to 5. Fixed electromorph differences at four loci, *AAT-L1*, *CK*, *GPI-2* and *SDH* allowed complete discrimination among the three Mediterranean populations. Indeed at Locus *GPI-2*, Portugal and western Mediterranean populations had different alleles at frequencies close to 1 (≥0.94). Some electromorphs were detected in single samples at frequencies large enough not simply to reflect a possible stochastic effect of sampling (Loci *AAT-L2*, *MPI* and *PGM*). Other electromorphs were not detected in one of the samples while being present at a substantial frequency in the other samples (Loci *AAT-L2*, *ADH*, *EST-L1*, *-L3*, *-4*, αGPDH-M and *ODH*).

Summary statistics on within-sample conformity with Hardy–Weinberg expectations as expressed by means of Weir and Cockerham's (1984) *f*-statistic are given in Table 6. As reported earlier (Galleguillos and Ward 1982), we found no indication of overall departure from the genotypic proportions expected under the null hypothesis of panmixia. For this, we compared the average *f* over loci to zero, assuming that *f* is normally distributed, by means of a two-tailed Student's *t*-test on *f*: SD

(jackknife); no f value was significantly different from zero. Single-locus f estimates were not significantly different from zero (χ^2 on genotype counts; Black and Krafsur 1985) except for *EST-L3* in ADRI (data per locus not shown).

Estimates of di-locus genotypic disequilibria were not significantly different from zero for all pairs of loci except *EST-L3/EST-4* ($p < 0.01$ in each of the four samples tested, namely TAJE, LIO6, ADRI and AEGE), which means that these two loci, and only these, could not be considered as statistically independent markers of population structure. Hence, *EST-L3*, which also showed stronger-than-average deviations from Hardy–Weinberg expected genotypic proportions, was removed from the subsequent analyses of population structure.

The results of the analysis of population structure at different geographic levels are summarised in Table 6.

Table 3 *Platichthys flesus*. Electromorph frequencies at 7 to 8 polymorphic enzyme loci in samples from European Atlantic coast [*Sample* (*locality*) *abbreviations* as in Table 1; (*N*) sample size]. Here and in Table 4, designations in parentheses are homologous electromorphs in Galleguillos and Ward (1982)

Locus, electromorph	Sample										
	LÜBE	KIEL	DENM	HELG	SCOT	SNOR	BRIT	PORT	COXT	SANJ	SETU
AAT-L2											
100	0.88	0.92	0.95	0.88	0.96	0.82	0.90	0.70	0.78	0.68	0.76
85	0.02	0.03	0	0.03	0	0.06	0.10	0.10	0.06	0.20	0.14
80	0	0.01	0	0	0	0	0	0	0.02	0	0
65	0.10	0.04	0	0	0.02	0.06	0	0	0.14	0.12	0.10
30	0	0	0.05	0.09	0.02	0.06	0	0.20	0	0	0
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(10)	(10)	(46)	(35)	(25)
EST-L3											
100	0.56	0.68	0.55	0.57	0.73	0.76	0.55	0.45	0.54	0.56	0.44
96	0.36	0.32	0.33	0.34	0.20	0.18	0.30	0.35	0.26	0.35	0.37
91	0.06	0	0.06	0.03	0.04	0.05	0.10	0.10	0.10	0.03	0.02
87	0.02	0	0.06	0.06	0.03	0.01	0.05	0.10	0.10	0.06	0.17
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(10)	(10)	(45)	(35)	(24)
EST-4											
110	0	0.02	0	0.01	0.03	0	0	0.07	0	0	0
100	0.94	0.86	0.89	0.85	0.85	0.88	0.81	0.70	0.84	0.88	0.76
96	0	0	0	0.01	0.01	0	0	0.07	0.01	0	0.02
91	0	0.04	0	0.01	0.03	0.06	0	0.03	0.02	0.03	0
84	0.06	0.08	0.11	0.12	0.08	0.06	0.18	0.13	0.13	0.09	0.22
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(8)	(15)	(7)	(33)	(20)
αGPDH-M (*αGpdh-1*)											
115 (*45*)	0.44	0.41	0.50	0.40	0.47	0.42	0.38	–	–	–	–
100 (*40*)	0.52	0.59	0.50	0.60	0.51	0.56	0.56	–	–	–	–
80	0	0	0	0	0.01	0.02	0.06	–	–	–	–
60	0.04	0	0	0	0.01	0	0	–	–	–	–
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(9)	–	–	–	–
GPI-2 (*Pgi-2*)											
102 (*110*)	0.60	0.46	0.72	0.88	0.67	0.70	1	1	0.99	0.96	1
100 (*100*)	0.40	0.53	0.28	0.12	0.33	0.30	0	0	0.01	0.04	0
98	0	0.01	0	0	0	0	0	0	0	0	0
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(10)	(15)	(47)	(35)	(25)
IDH-L (*Idh-1*)											
130	0	0	0	0.01	0	0	0	0	0	0	0
118 (*118*)	0.02	0.04	0	0.01	0.02	0.05	0.07	0.03	0.05	0.07	0.04
100 (*100*)	0.96	0.96	1	0.98	0.98	0.94	0.93	0.97	0.95	0.93	0.96
65	0.02	0	0	0	0	0.01	0	0	0	0	0
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(10)	(15)	(47)	(35)	(24)
IDH-M (*Idh-2*)											
130	0	0	0	0	0	0	0	0	0.01	0	0.04
110	0	0.01	0	0	0	0	0	0	0	0.01	0
100 (*100*)	0.90	0.96	1	0.91	0.92	0.92	0.93	1	0.99	0.99	0.96
91 (*88*)	0.10	0.03	0	0.09	0.08	0.08	0.07	0	0	0	0
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(7)	(15)	(47)	(34)	(24)
PGM (*Pgm-1*)											
130	0	0.01	0	0	0	0	0	0	0	0	0
115 (*186*)	0.02	0.03	0	0.03	0.02	0.03	0	0	0	0	0
100 (*140*)	0.76	0.81	0.78	0.84	0.80	0.77	0.84	0.80	0.82	0.84	0.82
81 (*100*)	0.22	0.14	0.22	0.13	0.18	0.20	0.16	0.20	0.17	0.16	0.18
68	0	0.01	0	0	0	0	0	0	0.01	0	0
(*N*)	(25)	(37)	(9)	(34)	(33)	(54)	(10)	(15)	(47)	(35)	(25)

Table 4 *Platichthys flesus* and *P. stellatus*. Electromorph frequencies at 6 to 24 polymorphic enzyme loci in samples from Portugal, Mediterranean Sea, and Japan [*Sample* (*locality*) *abbreviations* as in Table 1; (*N*) sample size]

Locus, electromorph	Sample										
	TAJE	EBRO	LIO1	LIO2	LIO3	LIO4	LIO5	LIO6	ADRI	AEGE	JAPN
AAT-L1 (*Got-1*)											
120 (*110*)	0	–	–	–	–	–	–	0	1	1	0.07
100 (*100*)	1	–	–	–	–	–	–	1	0	0	0.93
(*N*)	(32)	–	–	–	–	–	–	(26)	(22)	(20)	(22)
AAT-L2											
146	0	0	0	0	–	0	0	0	0.05	0	0
132	0	0	0	0	–	0	0	0	0.36	0.78	0
100	0.72	0.90	0.92	0.86	–	1	0.92	0.92	0.59	0.22	0.91
85	0.08	0.10	0.08	0.10	–	0	0.07	0.08	0	0	0
80	0	0	0	0	–	0	0	0	0	0	0.09
65	0.20	0	0	0.04	–	0	0	0	0	0	0
(*N*)	(27)	(5)	(19)	(24)	–	(32)	(167)	(26)	(22)	(20)	(16)
ADA (*Ada*)											
100 (*100*)	–	–	–	–	–	–	–	1	0.98	1	–
86	–	–	–	–	–	–	–	0	0.02	0	–
(*N*)	–	–	–	–	–	–	–	(26)	(22)	(20)	–
ADH											
123	0.12	0	–	0	–	–	–	0	0.09	0.30	1
100	0.88	1	–	1	–	–	–	1	0.91	0.70	0
(*N*)	(13)	(3)	–	(21)	–	–	–	(25)	(22)	(20)	(16)
CK (*Ck-M*)											
100	0.97	–	–	–	–	–	–	1	0	0.97	1
94 (*92*)	0	–	–	–	–	–	–	0	1	0	0
88	0	–	–	–	–	–	–	0	0	0.03	0
77	0.03	–	–	–	–	–	–	0	0	0	0
(*N*)	(15)	–	–	–	–	–	–	(26)	(22)	(20)	(5)
EST-D (*Est-D*)											
F	–	–	–	–	–	–	–	0	0	0	0.13
100 (*100*)	–	–	–	–	–	–	–	1	1	1	0.87
(*N*)	–	–	–	–	–	–	–	(26)	(22)	(20)	(4)
EST-L1 (*Est-2*)											
115	0	–	–	0	–	–	–	0	0	0.02	–
110	0	–	–	0	–	–	–	0	0	0.28	–
107 (*102*)	0.47	–	–	0.50	–	–	–	0.23	0.98	0.70	–
100 (*100*)	0.53	–	–	0.50	–	–	–	0.77	0.02	0	–
(*N*)	(19)	–	–	(1)	–	–	–	(26)	(22)	(20)	–
EST-L3											
100	0.63	0.75	0.50	0.58	0.43	0.33	0.43	0.46	0.21	0.75	–
96	0.25	0	0.26	0.25	0.37	0.40	0.38	0.33	0	0.05	–
91	0.04	0.25	0.21	0.13	0.14	0.19	0.11	0.11	0.48	0.20	–
87	0.08	0	0.03	0.04	0.06	0.08	0.08	0.10	0.31	0	–
(*N*)	(34)	(2)	(19)	(24)	(131)	(32)	(164)	(26)	(22)	(20)	–
EST-4											
100	0.89	–	–	–	–	–	–	0.77	0.64	0.95	–
95	0	–	–	–	–	–	–	0	0	0.03	–
91	0.02	–	–	–	–	–	–	0	0	0.02	–
84	0.09	–	–	–	–	–	–	0.23	0.36	0	–
(*N*)	(32)	–	–	–	–	–	–	(26)	(21)	(20)	–
αGPDH-L (*αGpdh-2*)											
100 (*100*)	–	–	–	–	–	–	–	1	0.87	1	–
S (*79*)	–	–	–	–	–	–	–	0	0.13	0	–
(*N*)	–	–	–	–	–	–	–	(6)	(8)	(17)	–
αGPDH-M (*αGpdh-1*)											
115 (*45*)	0.45	–	–	–	–	–	–	0.44	0.14	1	0
100 (*40*)	0.55	–	–	–	–	–	–	0.56	0.86	0	1
(*N*)	(32)	–	–	–	–	–	–	(26)	(22)	(20)	(22)
GPI-1 (*Pgi-1*)											
133 (*120*)	0.02	0	–	0	–	–	–	0	0.02	0	0
100 (*100*)	0.98	1	–	1	–	–	–	1	0.98	1	1
(*N*)	(33)	(5)	–	(24)	–	–	–	(26)	(22)	(20)	(16)
GPI-2 (*Pgi-2*)											
102 (*110*)	0.94	0	0	0	0	0	0	0	0.98	1	1
100 (*100*)	0.06	1	1	1	1	1	1	1	0.02	0	0
(*N*)	(33)	(5)	(19)	(24)	(133)	(33)	(165)	(26)	(22)	(20)	(16)

(continued overleaf)

Table 4 (continued)

Locus, electromorph	Sample										
	TAJE	EBRO	LIO1	LIO2	LIO3	LIO4	LIO5	LIO6	ADRI	AEGE	JAPN
IDH-L (Idh-1)											
118 (118)	0.03	0.20	0	0	0	0.02	0	0	0	0	0
100 (100)	0.97	0.80	0.92	0.98	0.97	0.98	0.98	1	1	1	0.97
75	0	0	0.03	0	0.01	0	0.01	0	0	0	0.03
65	0	0	0.05	0.02	0.02	0	0.01	0	0	0	0
(N)	(33)	(5)	(19)	(23)	(133)	(33)	(164)	(26)	(22)	(20)	(16)
IDH-M (Idh-2)											
100 (100)	1	1	–	0.98	–	–	–	0.98	1	0.95	1
91 (88)	0	0	–	0	–	–	–	0	0	0.05	0
71	0	0	–	0.02	–	–	–	0.02	0	0	0
(N)	(33)	(4)	–	(24)	–	–	–	(26)	(22)	(20)	(21)
LDH (Ldh-1)											
100 (100)	1	–	–	1	–	–	–	1	1	0.80	1
66	0	–	–	0	–	–	–	0	0	0.20	0
(N)	(13)	–	–	(21)	–	–	–	(26)	(22)	(20)	(16)
MDH-M2 (Mdh-2)											
128	0	0	–	0	–	–	–	0.02	0	0	–
109	0	0	–	0	–	–	–	0.02	0	0	–
100 (77)	1	1	–	1	–	–	–	0.96	1	1	–
(N)	(13)	(5)	–	(24)	–	–	–	(26)	(22)	(20)	–
ME-2 (Me)											
105	–	–	–	–	–	–	–	0	0	0.03	0
100 (113)	–	–	–	–	–	–	–	1	1	0.97	1
(N)	–	–	–	–	–	–	–	(25)	(22)	(20)	(4)
MPI											
105	0	0	0	0.02	0	0	0	0.17	0.48	0.17	–
100	0.85	1	0.76	0.96	0.95	0.94	0.96	0.73	0.52	0.83	–
95	0.15	0	0.24	0.02	0.05	0.06	0.04	0.10	0	0	–
(N)	(20)	(1)	(19)	(24)	(113)	(33)	(162)	(26)	(20)	(20)	–
ODH (Odh)											
100 (100)	–	–	–	–	–	–	–	1	0.89	0.23	1
88 (75)	–	–	–	–	–	–	–	0	0.11	0.77	0
(N)	–	–	–	–	–	–	–	(25)	(22)	(20)	(6)
6PGDH (6Pgdh)											
100 (76)	0.97	1	–	1	–	–	–	1	1	1	1
78	0.03	0	–	0	–	–	–	0	0	0	0
(N)	(17)	(1)	–	(24)	–	–	–	(26)	(22)	(20)	(22)
PGM (Pgm-1)											
115	0	0	0.02	0	0.04	0.03	0.03	0	0	0	0
100 (140)	0.79	0.60	0.74	0.82	0.71	0.71	0.77	0.75	1	1	0.84
90	0	0	0	0	0	0	0	0	0	0	0.16
81 (100)	0.21	0.30	0.11	0.10	0.16	0.18	0.15	0.13	0	0	0
68	0	0.10	0.11	0.08	0.08	0.04	0.03	0.10	0	0	0
60	0	0	0.03	0	0.01	0.04	0.02	0.02	0	0	0
(N)	(26)	(5)	(19)	(24)	(131)	(30)	(164)	(26)	(22)	(20)	(16)
SDH (Sdh)											
120	0	–	–	–	–	–	–	0	0	0	0.53
100 (99)	1	–	–	–	–	–	–	1	1	0	0.47
60	0	–	–	–	–	–	–	0	0	1	0
(N)	(13)	–	–	–	–	–	–	(16)	(22)	(13)	(16)
SOD (Sod)											
100 (100)	1	1	0.97	0.98	0.97	0.95	0.97	0.96	1	1	1
30	0	0	0.03	0.02	0.03	0.05	0.03	0.04	0	0	0
(N)	(33)	(5)	(18)	(24)	(129)	(33)	(164)	(26)	(22)	(20)	(22)

We found no indication of within-region structure in the Atlantic or Golfe du Lion. Low but significant differentiation was found among subpopulations in the Adriatic Sea, while no significant differentiation was evident between subpopulations of the Aegean Sea, the Marmara Sea or the Black Sea. At the within-basin level, some structure was observed in the Atlantic and a high degree of differentiation occurred in the Mediterranean, as already expressed by the occurrence of fixed allelic differences between populations.

The analysis of pairwise genetic distances between subpopulations in the Atlantic indicated that these may follow a pattern of differentiation by distance, although the correlation was weak (Mantel's test; 100 permuta-

Table 5 *Platichthys flesus* and *P. stellatus*. Loci exhibiting sample monorphism in Portugal, Mediterranean Sea and Japan [*Sample (locality) abbreviations* as in Table 1; –no data]

Locus	Sample size						
	TAJE	EBRO	LIO2	LIO6	ADRI	AEGE	JAPN
AAT-3	15	–	–	26	22	20	–
AK (Ak)	15	–	–	26	22	20	16
EST-L2	–	–	–	26	22	20	–
FUM	–	–	–	26	22	20	5
GLO (Glo-1)	–	–	–	24	21	20	16
MDH-L	15	–	24	26	22	20	–
MDH-M1 (Mdh-1)	15	5	24	26	22	20	16
XDH (Xdh)	–	–	–	26	22	20	8

Table 6 *Platichthys flesus* and *P. stellatus*. Summary of genetic variation at different geographic scales [*f* over-loci average of Weir and Cockerham's (1984) *f* statistic, fixation-index estimate within subpopulations; G_{ST} Nei's (1973) gene-diversity statistic among subpopulations; *SD* jackknife estimate of standard deviation across loci; (*N*) average number of individuals per locus per sample; *s* number of subpopulations; *r* number of polymorphic loci scored in all subpopulations; *PS* present survey; *GW* Galleguillos and Ward (1982); *PS+GW* comparisons using data from both PS and GW with calculation of G_{ST} on pooled electromorph frequencies (see "Materials and methods – Analysis of enzyme polymorphism"); * $p < 0.05$; *** $p \ll 0.001$; right-tailed Student's *t*-test with $df = r - 1$ (using Bonferroni correction); –no data]

Geographic scale, population	$f \pm$ SD	$G_{ST} \pm$ SD	(*N*)	*s*	*r*	Source
Within-region						
Baltic Sea	0.062 ± 0.083	−0.002 ± 0.003^{NS}	(31.0)	2	7	PS
	–	−0.010 ± 0.003^{NS}	(37.2)	3	4	PS+GW
North Sea	−0.015 ± 0.034	0.039 ± 0.006*	(32.5)	4	7	PS
	–	−0.017 ± 0.009^{NS}	(34.2)	5	5	PS+GW
SW Britain	–	0.002 ± 0.001^{NS}	(47.3)	2	13	GW
Brittany	0.035 ± 0.126	–	(9.3)	1	8	PS
Portugal	0.038 ± 0.032	−0.042 ± 0.003^{NS}	(28.7)	5	6	PS
Western Mediterranean	0.039 ± 0.063	−0.052 ± 0.022^{NS}	(65.1)	6	4	PS[a]
	–	−0.166 ± 0.035^{NS}	(140.1)	2	7	PS[b]
Adriatic Sea	0.151 ± 0.058	–	(22.0)	1	12	PS
	–	0.047 ± 0.007*	(33.7)	3	12	PS+GW
Aegean–Black Sea	0.096 ± 0.107	–	(20.0)	1	11	PS
	–	0.137 ± 0.075^{NS}	(11.0)	3	5	PS+GW
Japan	−0.084 ± 0.013	–	(15.0)	1	6	PS
Among-regions within basin						
Atlantic		0.036 ± 0.009*	(82.1)	4	7	PS
		0.047 ± 0.016*	(101.8)	5	5	PS+GW
Mediterranean		0.483 ± 0.006***	(56.4)	3	21	PS[a]
		0.528 ± 0.005***	(78.3)	3	21	PS+GW
Among geographically isolated populations[c]						
		0.625 ± 0.008***	(73.9)	5	14	PS[a]
		0.565 ± 0.007***	(96.4)	5	18	PS+GW

[a] Golfe du Lion samples only
[b] Ebro vs Golfe du Lion
[c] Populations recognised as geographically isolated were Atlantic, Western Mediterranean, Adriatic, Aegean–Black Sea and Japan

tions; $0.13 < p < 0.15$). No correlation was found between genetic distance and geographic distance at the scale of the whole distribution of the flounders (Mantel's test; 100 permutations; $p > 0.20$).

The parsimony tree inferred from RFLPs on mtDNA featured three major groupings (phylads) of individual haplotypes, one including all three individuals from the Aegean Sea, a second including a proportion of the individuals from Golfe du Lion, and a third including the other individuals from Golfe du Lion together with all individuals from the Atlantic (Fig. 2). The major branching patterns of the tree in Fig. 2 were not altered by bootstrap resampling, thereby demonstrating its robustness with regard to the sampling of characters.

Discussion

Geographic structure

At the time of reproduction, adult *Platichthys flesus* migrate from coastal lagoons and estuaries and congregate offshore. Mass spawning occurs, and eggs and

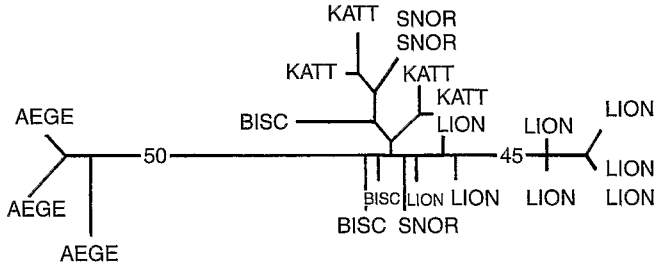

Fig. 2 *Platichthys flesus*. Unrooted parsimony tree inferred from restriction fragment-length polymorphism of individual mitochondrial DNA [*Numbers* indicate scores of unperturbed nodes out of 50 pseudotrees obtained by bootstrap resampling of characters; all other nodes had scores ≤31; *Sample* (*locality*) *abbreviations* as in Fig. 1 and Table 1; *LION* Golfe du Lion]

larvae presumably drift over large distances (Specchi and Scattaro-Miccoli 1980; Vianet 1985). As in the common sole *Solea vulgaris*, another coastal flatfish species whose genetic structure has been investigated using a multiple-geographic scale approach similar to the present one (Kotoulas et al. 1995a), populations at the scale of the North Sea region, for example, are expected to constitute panmictic or quasi-panmictic units. This is because of the high levels of gene flow that presumably occur each generation through the gathering of individuals from different areas for spawning, and the passive diffusion of eggs and larvae back to coastal and estuarine nursery areas (Koutsikopoulos et al. 1991; Kotoulas et al. 1995a). Our results for *P. flesus* accorded with that model, as there was no indication of within-population genetic structure at the regional scale.

Marine fishes in general tend to show little genetic subdivision between geographically separated populations (Gyllensten 1985; Ward et al. 1994). Gene flow, mediated by the passive transport of pelagic eggs or larvae or by adult migration over large distances, contributes to maintaining the genetic homogeneity of populations at a broad geographic scale. The slow pace of genetic drift in populations of large and stable effective size may also contribute to the maintenance of genetic homogeneity, even in the extreme case of geographic isolation – provided this is recent enough. The opposite case, i.e. significant genetic heterogeneity among populations separated by geographic distances presumably short enough to lie within the fishes' potential migration range, has also been reported (Lacson et al. 1989; Planes et al. 1994; Kotoulas et al. 1995b). In such cases, gene flow was inferred to be insufficient to counteract differentiation caused by genetic drift or differential selection. An intermediate pattern is when a species has a more or less continuous distribution across a range. Here, the balance between gene flow and the forces responsible for genetic differentiation in the long term may result in clines, whereby genetic differentiation increases with geographic distance.

Contrasting patterns of population structure were observed between *Platichthys flesus* populations of the Atlantic and the Mediterranean–Black Sea basin. Some genetic continuity, or more accurately, weak geographic differentiation was inferred from the geographic distribution of electromorph frequencies from the southwestern Baltic Sea to southern Portugal, while fixed allelic differences were observed between flounder populations from the Golfe du Lion, the Adriatic Sea and Aegean–Black Sea.

The low level of differentiation from the Baltic Sea to southern Portugal suggests that *Platichthys flesus* populations in the Atlantic are at present in equilibrium between a substantial level of gene flow and genetic drift. An estimate of gene flow can be derived from the estimate of G_{ST} by the approximation $N_m \approx (1-G_{ST}):4G_{ST}$ (Slatkin 1993), where N is the effective population size and m is the migration rate, giving $Nm = 5.1$ [with *SD* confidence interval = (3.7 to 7.8)]. This value is much lower than that ($Nm = 92.3$ to ∞ depending on locus) inferred from $0 \equiv G_{ST}$) estimates in *Solea vulgaris* (Kotoulas et al. 1995a), a result that could point to differences in the rate of genetic exchange among populations in these two flatfish species. However, it should be noted that the sampling design in *S. vulgaris* did not include samples from southwestern Europe. The *P. flesus* population from Portugal, whose geographic distance from other populations in the Atlantic (Baltic Sea, North Sea, Brittany) was one order of magnitude higher than distances within the latter group, largely contributed to the total within-Atlantic differentiation observed in the G_{ST} analysis.

Fixed-allele differences between populations indicated that the flounder in the Mediterranean Sea, including the Black Sea, consisted of three geographically isolated entities. Allozyme analyses also demonstrated the existence of a geographic barrier between southern Portugal (i.e. West of Gibraltar Strait) and Ebro Delta/Golfe du Lion (East of Gibraltar Strait), as expressed by the frequencies of different alleles (102 and 100, respectively) at Locus *GPI-2* that are nearly fixed on the different sides of Gibraltar Strait. Assuming the selective neutrality of allozyme polymorphisms, such a result indicates that gene flow through the Gibraltar Strait region has been interrupted for a long time. Nei's genetic distance between Atlantic and western Mediterranean flounder populations, based on genetic variation at 22 allozyme loci, was estimated as ~0.055. This value is comparatively high for populations within the same currently recognised subspecies, *Platichthys flesus flesus* (see Avise and Aquadro 1982; Nei 1987). Galleguillos and Ward (1982) showed that the flounders from the Adriatic Sea were genetically differentiated enough from the populations of the Black Sea ($D = 0.139 \pm 0.063$) to rank them as different subspecies, therefore confirming Norman's (1934) earlier taxonomy (*P. flesus italicus* in the Adriatic Sea, and *P. flesus luscus* in the Black Sea). Here, we observed a similar degree of divergence between flounder from the Adriatic Sea and from the Aegean Sea ($D = 0.134 \pm 0.047$) while no significant genetic differentiation was evident between the latter and *P. flesus luscus* from the Marmara Sea/

Black Sea. Hence we considered the whole Aegean Sea–Marmara Sea–Black Sea region to harbour a single population, geographically isolated from the neighbouring Adriatic Sea. The third geographically isolated population was that of the western Mediterranean, currently recognised as *P. flesus flesus*. Because of the large genetic difference with the neighbouring population from Portugal (clearly indicated by the nearly diagnostic locus *GPI-2*), we suggest that this population be given a separate status from the Atlantic *P. flesus flesus*. This view was previously held by Moreau (1892), but was not followed up by later investigators.

Mitochondrial DNA patterns of geographic structure were in accordance with allozyme patterns in that significant mtDNA differentiation was observed between the Atlantic Ocean and the Aegean Sea, while there was no indication of such differentiation among populations within the Atlantic region. Also, two groups of haplotypes were scored in the western Mediterranean, one specific to that region and strongly differentiated, the other apparently undifferentiated from the haplotypes characteristic of Atlantic samples. The occurrence of two unrelated phylads in the western Mediterranean is compatible with the hypothesis of a two-step colonisation of this region proposed at the end of this discussion.

Temperature, development, and gene flow

The Mediterranean flounder populations, which are confined to the coastal waters of the north of the western Mediterranean, the Adriatic Sea and the Aegean Sea, and the population from the coast of Portugal, are geographically separated from their neighbours by southward peninsulas, the coastal waters of which do not have monthly temperatures of < 14 °C (Robinson 1973). A review of the literature on the development of the early stages in flounders (references in Borsa 1986) indicates that the upper limit of temperature tolerance for eggs is ~12 °C. This value apparently does not vary among samples from the North Sea (Ehrenbaum 1909; Apstein *in* Russell 1976) and the Adriatic Sea (Varagnolo 1964). Spawning is seasonal (Altukhov 1980; Specchi and Scattaro-Miccoli 1980; Vianet 1985; Masson 1986 – and references therein). The spawning season varies according to locality, but occurs during the period when the sea-surface temperature is between 5 and 10 °C (maps of temperatures in Robinson 1973; Robinson et al. 1979). This is so over a large area from the White Sea, the northernmost location where *Platichthys flesus* occurs, to the Azov Sea (Grout 1984), its most easterly range. Although it is not clear which proximal factors, biotic or abiotic, trigger spawning in flatfishes, there is some indication that the onset of spawning migration is determined by a change in temperature (Van der Land 1991). Water temperature thus appears to be a likely determinant of spawning in *P. flesus*. It also appears that the temperature tolerance of the early planktonic stages is a limiting factor in both their southward dispersal capacities and the range that populations can occupy perennially.

Biogeographic implications

Clues on the biogeographic relationships of the NE Atlantic/Mediterranean marine faunas can be gathered from genetic studies on various species of fishes and molluscs. These include the bivalves *Mytilus galloprovincialis* (Quesada et al. 1995a,b), *Ostrea edulis* (Saavedra et al. 1993) and *Ruditapes decussatus* (Borsa et al. 1994); the anchovy *Engraulis encrasicolus* (Bembo et al. 1995, 1996; Magoulas et al. 1996); the flatfishes *Platichthys flesus* (Galleguillos and Ward 1982; present study), *Scophthalmus maximus* and *S. rhombus* (Blanquer et al. 1992) and *Solea vulgaris* (Kotoulas et al. 1995a); and the swordfish *Xiphias gladius* (Kotoulas et al. 1995b). No genetic differentiation was observed in *Scophthalmus rhombus*. No clear-cut geographic structure, but rather differentiation by distance, was reported in *R. decussatus* and *Solea vulgaris*, a pattern interpreted as the result of long-term equilibrium between gene flow and genetic drift (Slatkin 1993). Sharp genetic differences were observed, but these displayed different patterns: (1) in *M. galloprovincialis* and *X. gladius*, there were discontinuities between the Atlantic and the Mediterranean populations but not among the Mediterranean populations; (2) in *E. encrasicolus*, *O. edulis* and *Scophthalmus maximus*, there were differences among the western and eastern Mediterranean populations but not between the Atlantic and western Mediterranean populations. Thus, a proportion of species presently occurring in both the Atlantic and the Mediterranean have undergone an interruption in gene flow between adjacent populations. The geographic barriers apparently coincide with the two major oceanographical discontinuities of the area: the Gibraltar Strait–Alboran Sea region, and the Siculo–Tunisian Strait (see Ovchinnikov 1966). Different species do not necessarily exhibit the same patterns of geographic structure, probably as a result of different patterns of colonisation. It is worth noting that the more temperature-tolerant the early planktonic stages are, the weaker the geographic structure in flatfish populations (discussed in Kotoulas et al. 1995a).

The geographic structure of the flounder, consisting of four geographically isolated populations in the NE Atlantic/Mediterranean, thus revealed three discontinuities, in the Gibraltar Strait–Alboran Sea and the Siculo–Tunisian Strait plus a third hiatus between the Adriatic Sea and the Aegean Sea (the Peloponnese Peninsula). The UPGMA dendrograms in Fig. 3 show the presumed phylogenetic relationships between geographically isolated populations of *Platichthys flesus* and *P. stellatus*. The dendrogram of Fig. 3a was constructed using the genetic distances calculated on the maximum number of loci in pairwise comparisons (16 to 32 loci), while the dendrogram of Fig. 3b shows the genetic distances on the 16 loci common to all popula-

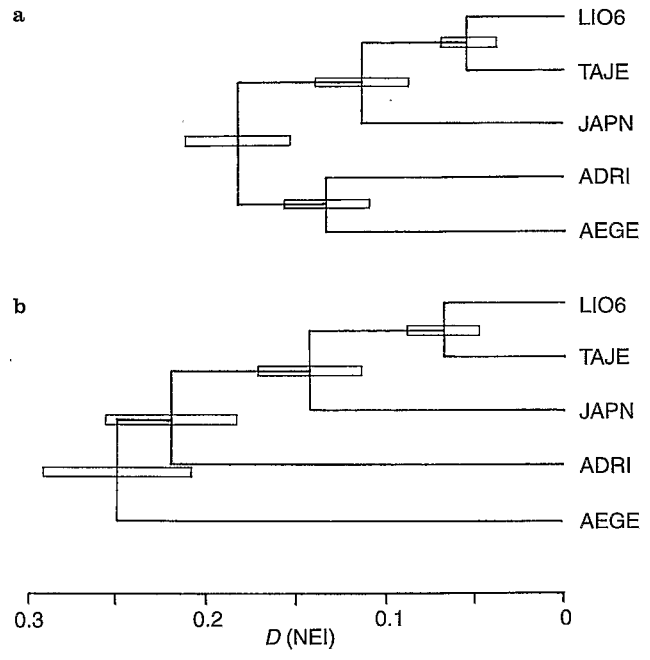

Fig. 3 *Platichthys flesus* and *P. stellatus*. Hypothetical phylogenetic relationships between geographically isolated populations. **a** UPGMA dendrogram based on Nei's genetic distances from electromorph-frequency data at 16 to 32 loci. **b** UPGMA dendrogram based on Nei's genetic distances from data at 16 enzyme loci surveyed in all populations (*AAT-L1*, *AAT-L2*, *ADH*, *AK*, *CK*, *αGPDH-M*, *GPI-1*, *GPI-2*, *IDH-L*, *IDH-M*, *LDH*, *MDH-M1*, *6PGDH*, *PGM*, *SDH* and *SOD*) [*D*(*NEI*) Nei's (1972) genetic distance between populations; *open rectangles* standard error on each side of branching point (Nei 1987); *sample* (*locality*) *abbreviations* as in Table 1]

tions. The biogeographic scenario proposed for *P. flesus* by Galleguillos and Ward (1982) using partially different samples and a partially different set of loci will not be discussed again here since, using the same assumptions of a linear relationship between genetic distance and time we reached the same conclusions of the early isolation of Atlantic *P. flesus flesus*, *P. flesus luscus* and *P. flesus italicus* from each another (Fig. 3). The following discussion will emphasize the new information provided by the analysis of samples from Portugal, Golfe du Lion, the Aegean Sea and Japan. The genetic divergence between the flounder populations from the Pacific Ocean (*P. stellatus*) and the Atlantic *P. flesus flesus* appears to be more recent than the event that led to the isolation of the proto-*P. flesus italicus*/*P. flesus luscus* population (Fig. 3). We therefore presume that the latter group became first isolated from the rest of the species, perhaps as early as 3.5 ± 0.6 million years ago as inferred from our UPGMA estimates of genetic distance and assuming the equivalence 1 unit of distance ≡ 19 million years (Vawter et al. 1980; Galleguillos and Ward 1982) is correct. We further propose that the remaining proto-*P. flesus flesus*/*P. stellatus* population still occupied a geographic area encompassing the Arctic Ocean coast of Eurasia until about 2.2 ± 0.5 million years ago, when the geographic isolation of the proto-*P. stellatus* population from the proto-*P. flesus flesus* population began. We presume this was caused by the drastic decrease in temperature that is known to have occurred at that time (Crame 1993), especially at high latitudes (e.g. Culotta 1993). Genetic differentiation has since increased between the proto-*P. flesus flesus* and the proto-*P. stellatus* populations. During this period, the proto-*P. stellatus* population moved towards lower latitudes in the Pacific while the proto-*P. flesus flesus* population moved towards the south in the northeastern Atlantic until it eventually invaded the western Mediterranean, perhaps merging with a differentiated paleo-*P. flesus* population that was still occupying the Golfe du Lion. The latter hypothesis is supported by the occurrence in the western Mediterranean of two mtDNA phylads and by their phylogenetic relationships to the Atlantic and the Aegean mtDNA haplotypes. No such secondary contact occurred between *P. flesus flesus* and the already differentiated *P. flesus italicus* and *P. flesus luscus*. The current western Mediterranean *P. flesus flesus* eventually also became geographically isolated from the Atlantic population. No gene flow appears to have occurred between these two regions for a geological time that may well be as much as 1 My, despite further glacial episodes up to as recently as 18 000 yr ago (Thiede 1978), expected to have facilitated contacts between populations.

In summary, the genetic relationships between the Atlantic and the western Mediterranean *Platichthys flesus* populations have now been investigated using a range of samples from the coastline around western and southern Europe. The present results extend and complement those of earlier surveys of geographic variation in flounders (Galleguillos and Ward 1982; Berrebi et al. 1985; Berrebi 1988). The species *P. flesus* appears to be paraphyletic, and the genetic differentiation between the western Mediterranean and the Atlantic *P. flesus flesus* populations seems to be large enough to recognise them as separate subspecies. The Gibraltar Strait, the Siculo-Tunisian Strait and the Peloponnese Peninsula (but not the Dardanelles Strait between the Aegean Sea and the Marmara–Black Sea) are inferred to be geographic barriers. The sea-surface temperature is presumed to be a limiting factor in the migration of the early planktonic stages. The geographic population structure of the flounder in the Mediterranean has been found to be divided to a degree previously unreported in the literature on marine fish with long-lived planktonic eggs and larvae (see Ward et al. 1994). We expect that the patterns of geographic structure of other coastal pelagic fish species which are also remnants of a boreal Mediterranean fish fauna (e.g. the skate *Raja clavata* and the sprat *Sprattus sprattus*; Ekman 1968; Quignard 1978) will be similarly complex.

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