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Discord in the family Sparidae (Teleostei): divergent phylogeographical patterns across the Atlantic–Mediterranean divide

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Abstract

The Strait of Gibraltar has been proposed to be the divide between two marine biogeographical regions, the Mediterranean Sea and the Northeast Atlantic. Intraspecific studies have shown, for several of the examined species, a reduction of gene flow between the two basins. The present study examines genetic variation at nuclear and mitochondrial loci in five marine teleost species belonging to the family Sparidae. Four samples for each species were analysed spanning the Northeast Atlantic and the Mediterranean. For all individuals 17 allozyme loci were scored and a combined single strand conformation polymorphism-sequencing approach was used to survey approximately 190 bp of the mitochondrial DNA (mtDNA) *D-loop* region. All five species share similar biological features. For three species, namely *Lithognathus mormyrus*, *Spondyliosoma cantharus*, and *Dentex dentex*, large mtDNA divergence was observed between Atlantic and Mediterranean samples. Little or no mtDNA differentiation was found in the other two species, *Pagrus pagrus* and *Pagellus bogaraveo*. Allozyme data revealed strong differentiation when comparing Atlantic and Mediterranean samples of *L. mormyrus* and *D. dentex*, moderate for *P. pagrus*, and no differentiation for *P. bogaraveo* and *S. cantharus*. These results provide evidence for a sharp phylogeographical break (*sensu* Avise) between the Atlantic and the Mediterranean for two (or possibly three) sparid species of the five investigated. At the same time, the obtained results for the other two species raise the question on which ecological/historical factors might have caused the observed discrepancy in the geographical distribution of genetic variation among otherwise biologically similar species.

Introduction

'… When at that narrow passage we arrived
Where Hercules his landmarks set as signals,
That man no farther onward should adventure.
On the right hand behind me left I Seville,
And on the other already had left Ceuta…'
(Dante Alighieri, Commedia, Inferno Canto XXVI)

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In his major work (composed during the years 1304–1321), the Italian poet Dante Alighieri set the unsurpassable limits of the known world at the Gibraltar Straits, the narrow passage connecting the Atlantic Ocean and the Mediterranean Sea. Since then, man has actually adventured farther on, yet for other species this connection still represents a boundary, although not an impassable one. The Strait of Gibraltar has been proposed to be the divide between two marine biogeographical regions, the Mediterranean Sea and the North-East Atlantic (Quignard, 1978), and additional evidence for this hypothesis has recently been provided by studies on

intraspecific genetic variation in a variety of organisms (Borsa *et al*., 1997; Pannacciulli *et al*., 1997; Naciri *et al*., 1999; Perez-Losada *et al*., 1999; Zane *et al*., 2000). These studies have shown, for several species, a reduction of gene flow between the Mediterranean and the Atlantic. A clear phylogenetic break [*sensu* Avise (1994)] has never been observed, and a few species show no differentiation at all between Atlantic and Mediterranean populations. Moreover, results for different species are often difficult to compare because of the use of different molecular markers and/or sampling schemes. Therefore, in the present study we carried out a comparative study on five closely related fish species. In addition, the same markers and sampling design were used to survey population genetic variation. Such an experimental strategy should warrant a better comparability across species. This in turn might be helpful for a better understanding of the role of the Atlantic–Mediterranean divide on marine species.

In other geographical areas, comparative phylogeographical studies have often disclosed concordant patterns across unrelated species (Avise, 1998a). Such common patterns are attributed to a shared history of fragmentation in the distribution of species, which causes long-term population subdivision. In the marine realm, a high potential for dispersal and/or absence of strong barriers to migration are believed to warrant high connectivity even between distant populations, and to prevent long-term population subdivision. This holds true especially for those species with a pelagic stage during their life cycle. Although there is increasing evidence that effective dispersal in the sea might not be as high as expected (Palumbi, 2000), examples of clear phylogeographical boundaries in marine taxa remain limited to the well-documented cases of the divide between the Gulf of Mexico and the Western Atlantic Coast (reviewed in Avise, 1994), and the separation between the Indian Ocean and the Western Pacific Ocean (Williams & Benzie, 1998).

We present here a study on intraspecific genetic variation in five marine fish of the family Sparidae (seabreams). This perciform family consists of more than 100 species, with a maximum of diversity in the northeast Atlantic and the Mediterranean region, where 24 species, in 11 genera, have been described (Bauchot & Hureau, 1986). Sparids are demersal fishes that are commonly found at variable depths (0–250 m) in temperate and tropical marine waters. Population samples of five species (*Dentex dentex* L., *Lithognathus mormyrus* L., *Pagellus bogaraveo* Brünnich, *Pagrus pagrus* L., *Spondyliosoma cantharus* L.) were collected from three geographical areas: the Northeast Atlantic immediately outside the strait of Gibraltar, and the Western and the Eastern Mediterranean Sea. After scoring genetic variation at nuclear and mitochondrial loci, the existence of a *bona fide* phylogeographical boundary at Gibraltar is conclusively supported for at least two, possibly three sparids. At the same time, the obtained results for the remaining species raise the question which ecological/historical factors might have caused the observed discrepancies in the geographical distribution of genetic variation among the five species examined in the present study.

Materials and methods

Collection of samples

Adult individuals were collected from different locations as shown in Fig. 1 and Table 1. Individuals were shipped on dry ice to the laboratory, where they were kept at −40 °C. Eye, liver and muscle tissue were removed for protein and DNA analyses. Because of technical difficulties, for two population samples (*P. bogaraveo*, Italian and Portuguese samples) specimens scored for allozyme variation could not be the same ones analysed for mitochondrial DNA (mtDNA).

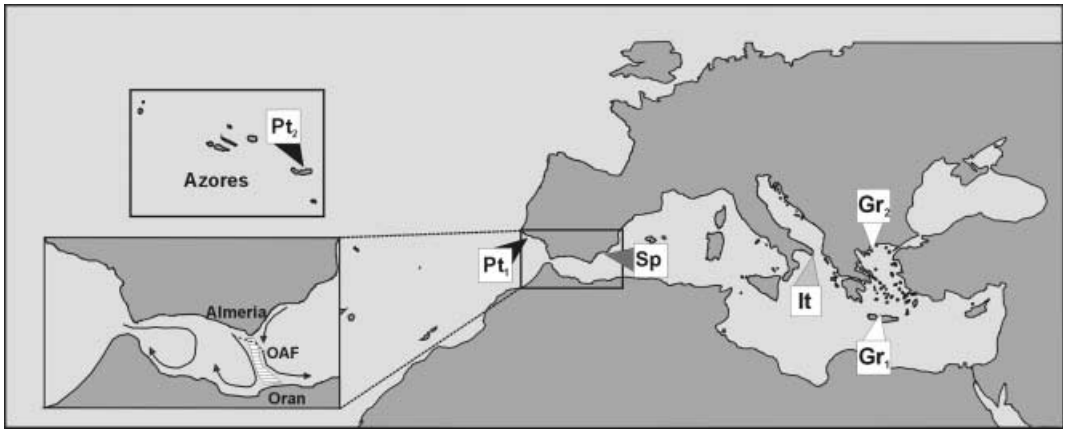

Fig. 1 Sampling sites. Samples were collected by or through local fishermen in Faro (Portugal, Pt_1), Azores (Portugal, Pt_2), Alicante (Spain, Sp), Otranto (Italy, It), Iraklion (Greece, Gr_1), North Aegean Sea, (Greece, Gr_2). The circulation in the Alboran Sea and the location of the Orian–Almeria Front (OAF) are also shown (see box).

Table 1 Sampling scheme and sample sizes. Sampling locations abbreviated as in Fig. 1.

	Sampling locations					
Species	Pt_1	Pt_2	Sp	It	Gr_1	Gr_2
Spondyliosoma cantharus						
mtDNA	18		19			14
Allozymes	40		23			
Dentex dentex						
mtDNA	30		28	23	45	
Allozymes	30		29	23	25	
Lithognathus mormyrus						
mtDNA	19		30	25	23	
Allozymes	20		31	25	26	
Pagellus bogaraveo						
mtDNA		29	48	25		29
Allozymes		30	49	27		29
Pagrus pagrus						
mtDNA	42		35	31	54	
Allozymes	46		38			

Allozymes

Experimental protocols were as in Alarcon & Alvarez (1999). Scored loci were: adenilate kinase (AK*), alcohol dehydrogenase (ADH*), esterase (EST), glucosephosphate isomerase (two loci: GPI-1*, GPI-2*), glycerol-3-phosphate dehydrogenase (G3PDH*), lactate dehydrogenase (three loci: LDH-1*, LDH-2*, LDH-3*), malate dehydrogenase (two loci: MDH-2*, MDH-3*), malic enzyme (two loci: MEP-1*, MEP-2*), phosphoglucomutase (PGM*), 6-phosphogluconate dehydrogenase (PGDH*), superoxide dismutase (two loci: SOD-1*, SOD-2*).

MtDNA

Total genomic DNA extraction, polymerase chain reaction amplification of mtDNA *D-loop* region, and single strand conformation polymorphism (SSCP) analyses were performed as described in Ostellari *et al.* (1996). Each sample was run two to three times under SSCP identical conditions. A few individuals from each mobility group (one to 10, depending on SSCP mobility class frequency) were randomly chosen, and their nucleotide sequences were determined using an automatic DNA sequencer (Vistra 725; Amersham Bioscience, Amersham, UK). The use of an internal primer and the fact that the sequenced region was larger than the SSCP fragment allowed unambiguously determination of the complete sequence of the region analysed by means of SSCP. All sequences were aligned using ClustalX (Thompson *et al.*, 1994), and individual sequences were re-examined manually by visual inspection of raw fluorogram-data. No discrepancies were observed between SCCP results and sequences. SCCP mobility classes were therefore treated as representative of sequence haplotypes.

The length of the *D-loop* region fragment that was analysed for each individual ranged between 190 and 210 bp, depending on the species.

For a single species, *L. mormyrus*, an additional mtDNA fragment was analysed: 193 bp of the cytochrome *b* (cyt-*b*) gene were scored by means of SCCP as described above, with species-specific primers (primer sequence is available upon request to the corresponding author). Sequencing, alignment and sequence analysis of the cyt-*b* gene were carried out as for *D-loop* fragments. All individuals scored for *D-loop* were also examined for variation at the cyt-*b* gene.

Analysis of genetic variation

Allozymes

Diversity indexes were calculated using the program Arlequin 2.000 (Schneider *et al.*, 2000), and Genetix 4.0 (http://www.univ-montp2.fr/~genetix/genetix.htm). For allozyme data the following indexes of genetic variability were estimated: number of polymorphic alleles (99% criterion), mean number of alleles per locus, observed heterozygosity (Ho) and expected heterozygosity (He) (Nei, 1987), over all loci. Arlequin was used to test for deviations from Hardy–Weinberg equilibrium, performing an exact test for multiple alleles (Guo & Thompson, 1992).

Differentiation between population samples was tested through an exact test of population differentiation, which was conducted taking into account genotypic frequencies (Goudet *et al.*, 1996). Genetic heterogeneity among populations was evaluated in pairwise comparisons as well as for all population samples taken together.

F_{st} values (single locus and multilocus) were estimated both for population pairs and for all populations, using a weighted analysis of variance (Weir & Cockerham, 1984). To test whether F_{st} values were significantly different than those expected under the null hypothesis of genetic homogeneity, a nonparametric approach was used as implemented in Arlequin with 100 000 permutations (reshuffling individuals among populations).

MtDNA

For each population sample as well as for all samples together, we estimated the haplotype diversity (h) and nucleotide diversity (π) (Nei, 1987). Genetic differentiation between population samples was evaluated implementing an exact test in Arlequin as already described for allozyme data. Alternatively, F_{st} values were estimated both for population pairs and for all populations taken together, using a weighted analysis of molecular variance (Excoffier *et al.*, 1992). Genetic distances between alleles

were estimated as uncorrected proportion of nucleotide differences. Significant deviations of F_{st} values from values expected under the null hypothesis of genetic homogeneity were assessed as described above for allozyme data. Both analyses were performed using Arlequin.

Phylogenetic relationships among haplotypes were reconstructed in the form of a 'reduced median network' (MN) (Bandelt *et al.*, 1995) using the program Network (ver. 2.0b, http://www.fluxus-engineering.com). Insertion–deletion events were recoded as transversions (TV).

MtDNA sequence data were also evaluated to test for departures from mutation-drift equilibrium, using Tajima's *D* statistics (Tajima, 1989). Significance was assessed by generating random samples under the hypothesis of selective neutrality and population equilibrium.

For both allozyme and mtDNA data analyses, sequential Bonferroni correction (Rice, 1989) was applied to correct for multiple tests.

Results

Allozyme data

Analyses of allozyme loci revealed that the proportion of polymorphic loci varied across populations and species (Table 2), as reflected in the average number of observed alleles per locus. Likewise, mean He ranged from 0.1 (*P. pagrus*, Spanish sample, SP) to 0.007 (*L. mormyrus*, Greek sample, GR). The results appear not to be related with sample size. The five species also differed in the degree of genetic differentiation among samples. *Dentex dentex* showed the most pronounced divergence between Atlantic and Mediterranean samples (Table 3), with F_{st} values near 1. This result stems from the presence, at seven allozyme loci (GPI-1, GPI-2, LDH-1, MDH-3, MEP-1, MEP-2, PGM), of alleles that are found nearly exclusively either in Mediterranean individuals or in Atlantic ones. Similarly high F_{st} values are observed in the comparison of the Portuguese sample (PT) of *L. mormyrus* with Mediterranean ones (Table 3). In this case, a single locus (G3PDH) presents alternative alleles, while little or no variation was observed for the other loci. In both *D. dentex* and *L. mormyrus*, no significant differentiation is present among Mediterranean populations.

For two species (*P. pagrus* and *S. cantharus*) only samples PT and SP were scored for allozymes. *Pagrus pagrus* shows a highly significant F_{st} (Table 3), although this result is determined by a single locus (PGDH), and all other polymorphic loci show no significant F_{st} values between population samples, while *S. cantharus* displays only a marginally significant differentiation (not significant after correction for multiple tests, Table 3), largely attributable to the PGM locus.

In the fifth examined species, *P. bogaraveo*, significant differences are observed only in a single pairwise comparison (Sp-It), whereas the Atlantic sample appears not to be genetically differentiated from the Mediterranean ones.

In summary, a wide variation was found in the degree of genetic divergence between the two marine basins, ranging from the extreme case of *D. dentex*, which shows evidence for a complete separation of the two areas, to the lack of differentiation of *P. bogaraveo*. This results is confirmed when a genotypic test of population differentiation is applied for each locus and pair of populations (data not shown).

Table 2 Genetic diversity at allozyme loci.

Species sample code	He* (SD)	*P*†	Average allele/locus
Pagrus pagrus			
Pt_1	0,0536 (0,1051)	0,353	1,4118
Sp	0,1079 (0,1713)	0,588	1,7647
Lithognathus mormyrus			
Pt_1	0,0087 (0,0259)	0,118	1,1176
Sp	0,0147 (0,0334)	0,177	1,1765
It	0,0177 (0,0499)	0,118	1,1176
Gr_1	0,0067 (0,0200)	0,118	1,1176
Dentex dentex			
Pt_1	0,0228 (0,0695)	0,235	1,2941
Sp	0,0212 (0,0526)	0,294	1,2941
It	0,0296 (0,0557)	0,294	1,2941
Gr_1	0,0173 (0,0545)	0,118	1,1176
Pagellus bogaraveo			
Pt_2	0,0355 (0,1218)	0,177	1,1765
Sp	0,0438 (0,1169)	0,353	1,3529
It	0,0451 (0,1207)	0,177	1,1765
Gr_2	0,0439 (0,1243)	0,235	1,2353
Spondyliosoma cantharus			
Pt_1	0,0217 (0,0577)	0,177	1,1765
S. cantharus			
Sp	0,0077 (0,0171)	0,177	1,1765

*Unbiased expected heterozygosity (He) calculated after Nei, 1987.
†Proportion of polymorphic loci under a 0.99 criterium (see Methods).
Abbreviations for population samples as in Fig. 1: Portugal-Faro (Pt_1), Portugal-Azores (Pt_2), Spain (Sp), Italy (It), Greece-Iraklion (Gr_1), Greece-North Aegean Sea (Gr_2).

Table 3 Pairwise (multilocus) F_{st} values calculated on allozyme frequency data.

Species	Pt-Sp	Pt-It	Pt-Gr	Sp-It	Sp-Gr	It-Gr
Dentex dentex	0.952**	0.943**	0.956**	0.011	−0.008	0.005
Lithognathus mormyrus	0.817**	0.813**	0.885**	−0.006	−0.006	0.007
Pagrus pagrus	0.135**					
Spondyliosoma cantharus	0.039					
Pagellus bogaraveo	−0.006	0.044	−0.003	0.068*	0.011	0.005

*$P < 0.05$, **$P < 0.0001$ after sequential Bonferroni correction.

MtDNA data

A variable number of haplotypes was found using SSCP and sequence analysis (accession numbers: AY014611–AY014768). As for allozyme variation, genetic diversity at the mtDNA level varied across species and populations (Table 4), with the lowest level of both nucleotide (π) and haplotype (h) diversity observed in *P. bogaraveo* Pt sample. The observed values of h and π are within the range of those reported in a review on mtDNA variation in 32 marine teleost species by Grant & Bowen (1998). In total, 190–210 bp of the *D-loop* region were surveyed in each species. In the case of *D. dentex*, the presence of a repeated motif in the 3′ part of the sequenced region of all Mediterranean individuals made the sequence alignment difficult. These data suggest that *D. dentex* is the species sampled which displays the highest degree of genetic differentiation between the two basins. This result was also observed for allozyme variation in this species. To examine this issue in a more rigorous context, the unalignable region was deleted and all further analyses performed on a reduced data set (98 bp).

In all species, a moderate compositional bias toward A–T (66–75%) was observed, in agreement with results for the same mitochondrial region in other teleost species (Meyer *et al.*, 1990, McMillan & Palumbi, 1997). Likewise expected was the substitution bias toward transitions (TS). No TV were observed between *P. pagrus* haplotypes, while TS : TV ratio ranged in the other species from 5 : 1 to 1.2 : 1. The lowest value was obtained comparing *D. dentex* haplotypes, which might indicate partial TS saturation given the large genetic distance between Atlantic and Mediterranean haplotypes.

Reconstruction of evolutionary relationships among haplotypes in the form of reduced median networks

Table 4 Mitochondrial DNA diversity values.

Species sample code	π*	h†	Scored‡ (sequenced) individuals per species	Number of haplotypes per species
Pagrus pagrus			120 (54)	32
Pt_1	0.006	0.42		
Sp	0.003	0.36		
It	0.003	0.35		
Gr_1	0.006	0.53		
Lithognathus mormyrus			95 (41)	23
Pt_1	0.017	0.62		
Sp	0.012	0.68		
It	0.016	0.90		
Gr_1	0.017	0.81		
Dentex dentex			126 (69)	23 (50)§
Pt_1	0.049	0.49		
Sp	0.008	0.56		
It	0.008	0.67		
Gr	0.018	0.66		
Pagellus bogaraveo			131 (48)	12
Pt_2	0.0004	0.069		
Sp	0.005	0.52		
It	0.004	0.42		
Gr_2	0.002	0.20		
Spondyliosoma cantharus			52 (52)	41
Pt_1	0.016	0.94		
Sp	0.019	0.98		
Gr_2	0.018	0.98		

*Nucleotide diversity (see Methods).
†Haplotype diversity (see Methods).
‡Number of specimens analysed by means of single strand conformation polymorphism.
§Number of haplotypes when considering the complete data set (see text).
Abbreviations for population samples as in Fig. 1: Portugal-Faro (Pt_1), Portugal-Azores (Pt_2), Spain (Sp), Italy (It), Greece-Iraklion (Gr_1), Greece-North Aegean Sea (Gr_2).

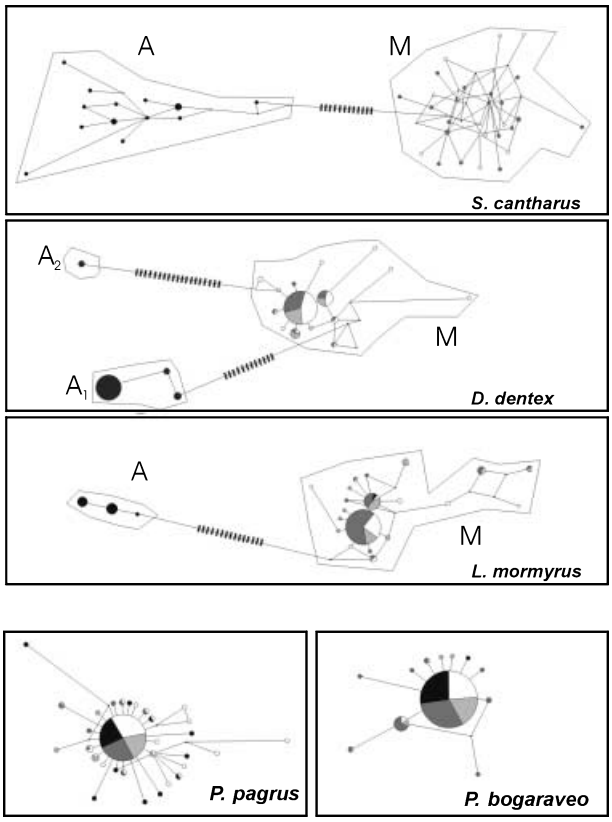

Fig. 2 Median networks based on mtDNA sequences. Circles represent haplotypes, with size proportional to relative frequencies. Branch lengths are proportional to number of substitutions, except for 'long branches' where relative numbers of changes are indicated as black bars. For each haplotype present in more than one population sample, sectors of different colors (black, Portugal; dark gray Spain; light gray Italy; white, Greece) refer to absolute number of haplotype counts for each population. Mediterranean (M) and Atlantic (A) clades are indicated.

Table 5 Pairwise 'molecular' F_{st} values calculated from mitochondrial DNA data.

Species	Total	Pt-Sp	Pt-It	Pt-Gr	Sp-It	Sp-Gr	It-Gr
Dentex dentex	0.753**	0.820**	0.811**	0.812**	−0.007	−0.013	0.000
Lithognathus mormyrus (D-loop)	0.741**	0.882**	0.861**	0.858**	−0.009	−0.016	−0.010
L. mormyrus (cyt-b)	0.918**	0.948**	0.931**	0.932**	0.008	0.006	0.000
Pagrus pagrus	0.003	0.009	0.001	0.000	−0.002	0.007	0.002
Spondyliosoma cantharus	0.786**	0.842**		0.844**		−0.032	
Pagellus bogaraveo	0.022*	0.075*	0.038*	0.045	−0.002	0.003	−0.025

*$P < 0.05$, **$P < 0.0001$ after sequential Bonferroni correction.

(MNs) confirms that the five examined species do not share a common pattern of genetic diversity (Fig. 2). For *L. mormyrus* the MN shows two groups of haplotypes (A and M) connected by a long branch (15 substitutions). As indicated by the colour code, only 'Atlantic' individuals are included in group A, while group M consists of 'Mediterranean' sequences, except for a single Atlantic specimen belonging to the second most frequent haplotype. For this species, a large divergence between Atlantic and Mediterranean samples is evident also when examining a different mtDNA fragment (cyt-*b*). Fewer polymorphic sites are found in the cyt-*b* region analysed, yielding a smaller number of haplotypes (six) when compared with the *D-loop*. A lower sequence divergence between alleles is expected for cyt-*b* which is known to be less variable than *D-loop*, with a substitution rate generally five to ten times lower (McMillan & Palumbi, 1997). Despite the low variation observed, F_{st} and population differentiation analyses give results that are identical to those obtained for the more variable *D-loop* region (Table 5). Likewise, in the reconstructed cyt-*b* MN (not shown) Portuguese and Mediterranean haplotypes are separated by a minimum of four substitutions.

Spondyliosoma cantharus is characterized by a similarly clear phylogenetic separation between Atlantic and Mediterranean haplotypes (with a minimum of 12 substitutions). The latter species also displays high haplotypic diversity (Table 4) with very few individuals sharing the same haplotype. A third species, *D. dentex*, presents a sharp phylogeographical break between the two marine basins, with the majority of Atlantic individuals falling into group A_1 and all Mediterranean samples into group M, with a minimal distance of 12 substitutions. In addition, a small group of sequences (A_2, three individuals), was found in the Atlantic sample, showing a large divergence from both group A_1 and M.

In contrast to the deep phylogenetic structure of *L. mormyrus*, *S. cantharus*, and *D. dentex*, the remaining two species, *P. bogaraveo* and *P. pagrus*, exhibit a 'shallow' mtDNA network, with a 'star-like' shape, characterized by several haplotypes, at low frequency, stemming from the most common one. No separation appears to be present between Atlantic and Mediterranean individuals.

Statistical support for the above pattern comes from the analysis of F_{st} values in global and pairwise population comparisons (Table 5). The same three species show a markedly structured haplotype network with extremely high global F_{st} values. Evidence from pairwise F_{st} values indicates that this result is caused by the strong genetic divergence between the Portuguese sample and the Mediterranean ones. Identical results, suggesting a strong Atlantic–Mediterranean differentiation for these three species, are obtained using an exact test of genotypic population differentiation (data not shown). No significant differentiation is ever observed between Mediterranean populations.

Estimated molecular distances between Atlantic and Mediterranean clades in *D. dentex*, *L. mormyrus*, and *S. cantharus* indicate long divergence times. Average distances are respectively 13%, 17%, and 16.4%. As mentioned earlier, the divergence between the *D. dentex* haplotype groups is likely underestimated, as a large (100 bp) unalignable region was not considered, and only the more conserved 5′ end was taken into account. To our knowledge, the observed divergence is the highest reported thus far for intraspecific differentiation between Atlantic and Mediterranean populations. If we assume a divergence rate of 11% per million years (Myr) for the *D-loop* in teleosts (McMillan & Palumbi, 1997), a separation time of the two clades could be inferred to be 1.2–1.5 Myr, depending on the species. This estimate is confirmed when examining cyt-*b* data for *L. mormyrus*. In this case, the average divergence between Atlantic and Mediterranean clades is 3.6%. Applying a conventional mtDNA rate of 2% per Myr (coding regions, all sites, uncorrected sequence divergence), divergence time is estimated to be 1.8 Myr. Moreover, the observed ratio between *D-loop* and cyt-*b* divergence is approximately 5 : 1 (17 : 3.6), in agreement with previous studies of marine fish [6.45 : 1, *D-loop*: total mtDNA in swordfish (Alvarado *et al.*, 1995), 4.3 : 1 *D-loop*: cyt-*b* in mackerel (Nesbo *et al.*, 2000)] suggesting a 10% per Myr rate for sparid *D-loop*, which is very similar to the one assumed above. Although large stochastic errors are potentially associated with estimates of divergence time, data from mtDNA variation in *D. dentex*, *S. cantharus*, and *L. mormyrus* suggest a scenario of allopatric separation between populations that belong to the two marine basins, dating back to early Pleistocene (1.2–1.8 Myr ago).

The remaining two species (*P. pagrus* and *P. bogaraveo*) are characterized by rather different patterns. The global F_{st} value estimated for *P. bogaraveo* is one order of

Table 6 Tajima's *D* values calculated on mitochondrial DNA data.

Species	Pt	Sp	It	Gr
Dentex dentex	−0.72	−1.37	−0.64	−2.28*
Lithognathus mormyrus	−1.99*	−0.64	−0.92	−0.89
Pagrus pagrus	−2.50***	−2.00**	−1.73*	−2.34**
Spondyliosoma cantharus	−1.59	−0.77	−1.04	
Pagellus bogaraveo	−1.14	−1.67	−1.69	−0.99

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, after sequential Bonferroni correction.

magnitude lower than the one observed for *L. mormyrus*, *S. cantharus*, and *D. dentex* (Table 5). This result seems to be attributable to marginally significant differences between the Portuguese sample (namely PT_2 from Azores) and the SP and IT samples. No significant deviation is observed when applying an exact test of population differentiation. For *P. pagrus*, neither significant F_{st} values nor instances of population differentiation are found.

To further evaluate the distributions of mtDNA diversity, Tajima's *D* statistics were estimated for each population, for all five species. This approach should allow the detection of departures from a mutation-drift equilibrium, because selective and other nonequilibrium processes are expected to shift the value of *D* toward positive or negative values. A few significant deviations from 'neutrality' were indeed observed (Table 6). This should be considered a conservative estimate because of the limited sample sizes. The most remarkable result is that all four *P. pagrus* samples indicate that a selective sweep or a population bottleneck occurred in the past with sufficient intensity or duration to leave a detectable trace in the present-day levels of mtDNA polymorphism.

Discussion

Thus far, genetic studies have yielded rather contradictory results about the hypothesis that the Western opening of the Mediterranean represents a phylogeographical boundary for marine species. For some species a clear differentiation is observed between Atlantic and Mediterranean populations, whereas for other species samples from the two basins appear genetically homogenous. These conflicting results, however, might be explained as a consequence of the use of different genetic markers (nuclear vs. mitochondrial loci, fast evolving vs. slow evolving loci) and the implementation of different sampling strategies. In the present work, however, the markers used and the sampling scheme adopted were strictly comparable. Nevertheless, the results obtained could not be reconciled with a univocal pattern. Experimental data from *D. dentex* and *L. mormyrus* lend strong support to the presence of a phylogeographical boundary between the Atlantic and the Mediterranean, whereas results from the analysis of genetic variation of *S. cantharus*, *P. pagrus*, and *P. bogaraveo* provide only limited evidence for a separation between the two basins.

Indeed, the results of *D. dentex* and *L. mormyrus* do conform to all three aspects of genealogical concordance that allow the definition of true phylogeographical boundary (Avise, 1998a): (i) concordance across multiple loci within a species, (ii) concordance in the geographical positions of significant gene-tree partitions across multiple co-distributed species, (iii) concordance of gene-tree partitions with boundaries between recognized biogeographical provinces.

(i) Concordance across multiple loci within a species: A relevant criticism on phylogeography is that its conclusions are often based on evidence from a single genetic marker, generally mtDNA. Concordant evidence from allozyme data allows rejection of such criticism, suggesting that mtDNA genealogies truly reflect deep historical partitions in the evolution of these two species. Morphometric analysis also revealed a marked difference between Atlantic and Mediterranean individuals of both species (J. Palma, personal communication). Moreover, the presence of three individuals of *D. dentex* in the PT_1 sample that belong to a third, divergent group of haplotypes is suggestive of additional complexity in this species. (ii) Concordance in the geographical positions of significant gene-tree partitions across multiple co-distributed species: MtDNA networks of *D. dentex* and *L. mormyrus*, as well as of *S. cantharus* are concordant in the partition of geographical samples into two separate phylogenetic groups. These species are found to show reciprocal monophyly between Atlantic and Mediterranean mtDNA haplotypes. Such concordance indicates that the same historical biogeographical factors likely influenced intraspecific patterns of genetic differentiation in these species. Additional evidence comes from estimates of divergence time between Atlantic and Mediterranean clades, suggesting that gene flow across the Gibraltar Strait in *D. dentex* and *L. mormyrus* has been low or nonexistent for the last 1.2–1.8 Myr. Such a temporal (early Pleistocene) and spatial (Gibraltar Strait) placement of a phylogeographical boundary is in agreement with geological data (Nilsson, 1982), which indicate that climate fluctuations along the entire Quaternary period have produced episodes of habitat fragmentation between the Atlantic and the Mediterranean. During glacial maxima the sea level recurrently dropped 115–120 m below the present-day level, reducing both width and depth of the Gibraltar Passage.

On a much shorter time scale, some hydrological features might have reduced gene flow between the two sides of the Strait. Oceanographical surveys have demonstrated that the inflowing Atlantic water describes a quasi-permanent anticyclonic gyre in the western Alboran Sea and a less stable one in the eastern part (Millot, 1999). The particular water circulation in the Alboran Sea generates an oceanographical front located from

Oran (Morocco) to Almeria (Spain), called the Oran–Almeria Front (OAF) (Fig. 1). It is unclear whether the present hydrological regime was similar also in the past, especially during the climatic changes mentioned. However, simulation studies suggest that the volume flow rate into the strait influences the growth rate of the gyre(s), but not its general structure (Gleizon *et al.*, 1996). Thus changes in the section of the Gibraltar Strait associated with climate modifications might have led to changes in levels of water flow, but not to reversal of hydrological conditions. The relevance of the OAF, in the present as well as in the past, is underscored by genetic evidence. Population genetics studies on sea bass (Naciri *et al.*, 1999), the Northern krill (Zane *et al.*, 2000), and the mussel *Mytilus galloprovincialis* (Quesada *et al.*, 1995), where samples from the Alboran Sea were included, demonstrate that a clear shift in gene frequencies is observed associated with the OAF. The concordant patterns found for *D. dentex* and *L. mormyrus* are consistent temporally (large divergence among haplotypes, reciprocal monophyly) and spatially (sharp change in genetic composition between Atlantic and Mediterranean samples) with the hypothesis that the historical and contemporary factors mentioned might have reduced gene flow between the two marine basins.

(iii) Concordance of gene-tree partitions with boundaries between recognized biogeographical provinces: The Strait of Gibraltar has traditionally, although not universally, been proposed as a transition zone between two biogeographical provinces, the Northeast Atlantic and the Mediterranean (Quignard, 1978). The phylogeographical break found within two sparid species appears to align geographically with this division, suggesting that historical factors shaping species composition in regional communities might be similar to those influencing geographical partition of populations within species.

In the case of *S. cantharus*, high divergence was observed between the Atlantic sample and the Mediterranean ones, with a clear phylogenetic separation of mtDNA haplotypes (Fig. 2). Allozyme loci, however, are nearly invariant especially in the Atlantic sample, not providing significant evidence for population differentiation. Therefore, the results from this species fail to conform to the first one of the three aspects of genealogical concordance. Although the pattern observed for mtDNA can hardly be reconciled with the hypothesis of high gene flow between Atlantic and Mediterranean, further studies on fast evolving nuclear loci are needed to confirm or reject the evidence from mtDNA data.

The results obtained from the two remaining species included in the present work are in stark contrast with the scenario delineated above. *Pagellus bogaraveo* shows a marginally significant differentiation between Atlantic and Mediterranean samples, only at the mtDNA locus, and limited to two pairwise comparisons (Table 5). *Pagrus pagrus* displays no differentiation at all for mtDNA, while at a single allozyme locus allele frequencies are significantly different between PT and SP samples. In both species, neither fixation of alternative alleles nor reciprocal monophyly of mtDNA haplotypes is observed, in contrast to the other three species analysed.

Incongruent phylogeographical results received far less attention than concordance in phylogeography across diverse taxa, although these observations may be important in revealing historical differences among species in gene flow, in sensitivity to barriers or selective gradients, in effective population size and other ecological and/or demographical factors (Zink, 1996; Avise, 1998b). Several hypotheses might be put forward to explain the concurrence of a phylogeographical break in *D. dentex*, *L. mormyrus*, and possibly in *S. cantharus*, together with little or absent differentiation in *P. pagrus* and *P. Bogaraveo*. Differences in measured levels of gene flow could originate from differences in dispersal and/or effective population size among species. Species showing no differentiation might have higher dispersal ability, or even if the capacity to migrate is generally comparable, each species might respond differently to the presence of oceanographical barriers, for instance through active migration across the barrier itself. All the species examined are reported to be sedentary as adults, thus dispersal is likely to occur mainly during the pelagic larval phase before settlement (Macpherson, 1998), although for most sparid species retention mechanisms have been hypothesized and probably only under exceptional conditions are larvae massively dispersed by hydrographical processes (Vigliola *et al.*, 1998). With regard to population size limited information suggests that census sizes should be comparable across species (Bauchot & Hureau, 1986). For mtDNA, some effect on long-term effective population size might be due to the peculiar mode of sex determination in sparids. Most sparid species are sequential hermaphrodites (Bauchot & Hureau, 1986), either proterandric (first male, then female) or proterogynic (first female, then male), leading to biased sex ratios in the most abundant year-classes. Again, the observed patterns of genetic differentiation appear not to correlate with reproduction mode, as *L. mormyrus* and *P. bogaraveo* are proterandric hermaphrodite, *S. cantharus* and *P. pagrus* proterogynic, and *D. dentex* generally has separate sexes. Unfortunately however, no direct measurements of dispersal capacity, especially in relation to oceanographical barriers, nor reliable estimates of effective population sizes are available for any sparid species. Likewise, data on sparid ecology are insufficient to decide whether only in two or three species different habitat requirements might have led to a distinct partition of individuals according to the diverse ecological features of the two basins. Therefore, although none of the available information on sparid biology (population size, life-history, ecology) seems to explain the divergent pattern observed, our current knowledge does

not allow to safely exclude any of the above mentioned hypotheses.

Alternatively, it might be hypothesized for *P. pagrus* and *P. bogaraveo* that, even if migration between contemporary populations of the Atlantic and the Mediterranean is effectively low or nonexistent as for the other sparid species, the absence of genetic differentiation is the consequence of historical events. For instance, if dramatic reductions in size brought Mediterranean populations to extinction, the lack of differentiation might be the exceptional result of a recent recolonization of the Mediterranean from the Atlantic. Under this scenario, there was not sufficient time for the populations of the two basins to diverge again, even if migration across the Gibraltar Strait is very limited, as suspected for other sparid species. For *P. pagrus* negative values of Tajima's *D*, all highly significant after Bonferroni correction (Table 6) could be explained as a consequence of a strong bottleneck and a subsequent expansion, in accordance with the hypothesis of extinction–recolonization. For *P. bogaraveo*, its distribution, which is limited to the western-most part of the Mediterranean basin might indicate that the presence of this species in the Mediterranean has been (re-)established only recently, without the possibility of accumulating detectable divergence between Atlantic and Mediterranean populations. The hypothesis of extinction–recolonization (or recent colonization) is also congruent with geological and paleo-oceanographical data. Although glacial periods likely reduced exchanges between the Mediterranean and the Atlantic, during interglacial periods, the sea level was suddenly raised, with the movement of large water masses, and possibly provided the opportunity to (re-)invade the Mediterranean basin. This explanation does not exclude the possibility of recurrent invasions might have occurred during interglacials without extinction of the Mediterranean lineage in other species. A pattern consistent with the latter scenario was found for *Diplodus puntazzo*, another Atlantic–Mediterranean fish species (L. Bargelloni & T. Patarnello, unpublished data).

In conclusion, although genetic markers and sampling strategy were strictly comparable, the results obtained for the five examined species appear to reflect, within a single fish family, the conflicting evidence on the Atlantic–Mediterranean divide that was available for other, much more diverse marine species. For two or three sparids there was a clear indication for the presence of a *bona fide* phylogeographical boundary at the Gibraltar Strait, but the observed differentiation pattern cannot be considered of general validity, not even for species with comparable ecological features, because it was not found in two or three other species. While these results underscore the importance of a comparative, multi-species approach in evaluating alternative phylogeographical hypotheses, it could not be reasonably established whether the observed divergent patterns are caused by present-day ecological differences between species or the consequence of historical events. Further studies on the biology of sparid (measuring dispersal) and better estimates of gene flow over short geographical ranges (using fast-evolving nuclear markers) are therefore needed to understand the observed differences.

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