MOLECULAR ECOLOGY

Molecular Ecology (2009) doi: 10.1111/j.1365-294X.2009.04404.x

Contrasting signals from multiple markers illuminate population connectivity in a marine fish

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Abstract

Recent advances in molecular biology and bioinformatics have helped to unveil striking and previously unrecognized patterns of geographic genetic structure in marine populations. Largely driven by the pressing needs of fisheries management and conservation, studies on marine fish populations have played a pivotal role in testing the efficiency of a range of approaches to explore connectivity and dispersal at sea. Here, we employed nuclear and mitochondrial DNA markers and parasitic infestations to examine the nature and patterns of population structure in a warm-temperate coastal marine teleost across major putative biogeographic barriers in the Mediterranean Sea and Eastern Atlantic Ocean. We detected deep genetic divergence between mitochondrial lineages, likely caused by dramatic climatic and geological transformations before and during the Pleistocene. Such long-diverged lineages later came into secondary contact and can now be found in sympatry. More importantly, microsatellite data revealed that these lineages, after millions of years of independent evolution, now interbreed extensively. By combining genetic and parasite data, we were able to identify at least five independent demographic units. While the different genetic and parasite-based methods produce notably contrasting signals and may complicate the reconstruction of connectivity dynamics, we show that by tailoring the correct interpretation to each of the descriptors used, it is possible to achieve a deeper understanding of the micro-evolutionary process and, consequently, resolve population structure.

Keywords: Atlantic Ocean, genetic structure, Indian Ocean, *Lithognathus mormyrus*, Mediterranean Sea, parasites, seascape genetics, Sparidae

Received 1 July 2009; revision accepted 17 September 2009

Introduction

Over the last two decades, advances in molecular biology and bioinformatics have helped to unveil previously unrecognized levels and patterns of geographic genetic structure in marine species (Peijnenburg *et al.* 2004; Hauser & Carvalho 2008). The heterogeneity in genetic structure broadly results from the complex interplay between life histories and environmental variation over space and time (Hauser & Carvalho 2008; Selkoe *et al.* 2008; Chopelet *et al.* 2009). Yet, the relative inaccessibility of the marine environment, the sheer magnitude of the spatial and temporal scales involved and the generally weaker underlying genetic structure compared to terrestrial landscapes (Hauser & Carvalho 2008; Selkoe *et al.* 2008), still makes investigating structure at sea a tougher challenge than on land.

Because of their historical importance to human economies, and their diversity of species, life histories, and behaviours, fish have played a pivotal role in the formulation of ideas on marine population structure, contributing to our increased understanding of phylogeographic breaks (Lessios & Robertson 2006; Patarnello *et al.* 2007), climate-driven demographic fluctuations (Lecomte *et al.* 2004; Gonzalez & Zardoya 2007), oceanographic barriers to dispersal (Galarza *et al.* 2009), local genetic adaptation (Williams & Oleksiak 2008), and fishery-induced evolution (Kuparinen & Merila 2007). The complexity of interactions among the above factors,

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along with the relentless pace at which oceans are being exploited and impacted, has made 'seascape' genetics and ecology one of the most fertile grounds of scientific inquiry (Selkoe *et al.* 2008).

The requirements of management and conservation policies have hitherto put great emphasis on the application of reliable and unambiguous methods for the identification of demographically independent population units (i.e. 'stocks'). Molecular genetic markers have become more frequently and extensively employed (Schwartz *et al.* 2006; Waples *et al.* 2008), especially in an interdisciplinary framework (McClelland *et al.* 2005; Koblmüller *et al.* 2007; Selkoe *et al.* 2008; Alpermann *et al.* 2009). The identification of population units and patterns of connectivity can in fact be greatly improved by the integration of genetic data with life history and ecological information (Ruzzante *et al.* 2006) as well as with physical and environmental modelling (Knutsen *et al.* 2004; Galarza *et al.* 2009). However, the simultaneous application of phenotypic and genetic methods as diverse as those currently available to marine population biologists very seldom results in a neat, consistent, unambiguous signal (Peijnenburg *et al.* 2006; Ruzzante *et al.* 2006; Abaunza *et al.* 2008). Furthermore, scientific and technological advancements are generally made at a faster pace than policies and legal procedures (Sagarin *et al.* 2009), resulting in rapidly changing operational scenarios that are often met with scepticism by policy makers and managers.

Different molecular markers can yield contrasting results because of different responses to evolutionary forces. For instance, neutral markers essentially examine the roles of gene flow and random drift, whereas markers that are under the influence of natural selection can inform us about local adaptive processes (Avise 2004). Contrasting signals can also be found when dealing with results obtained from both rapidly evolving nuclear noncoding markers (i.e. microsatellites) and nonrecombining maternally inherited markers (i.e. mitochondrial DNA, mtDNA). Microsatellites, for example, present high mutation rates and are able to unravel contemporary fine-scale population structure and investigate recent demographic events (Hewitt 2004); mtDNA may instead depict historical processes and/or offer an insight into sex-bias phenomena due to the nature of its inheritance (Avise 2000).

Life history and ecological traits can also effectively complement seascape genetic approaches. One particular class of natural tags employed to examine population connectivity are parasites (MacKenzie 2002). Parasitic faunas tend to vary between fish populations inhabiting different areas, which is often interpreted as evidence of demographic independence between stocks (MacKenzie & Abaunza 1998). However, host–parasite systems also constitute highly selective environments to which each component must adapt. The success of the parasites depends on both the strength of selection and the potential to adjust to new conditions set by the host (Gandon & Michalakis 2002). Thus, parasite faunas can provide synthetic information on both the nature of the demographic exchange among host populations, as well as the evolutionary differentiation caused by environmental gradients set by the host itself.

The present study pinpoints the advantages of using data from a suite of genetic and life history descriptors with different ecological and evolutionary characteristics (parasitic infection, mtDNA, and microsatellites) in providing a comprehensive picture of geographic population structure in a coastal marine fish. We chose as a study model the striped sea bream, *Lithognathus mormyrus* L., a commercially valuable species from the family Sparidae, which is commonly found on sandy bottoms across major traditional biogeographic breaks across the Mediterranean and the Eastern Atlantic. Sampling locations were identified to explore spatial genetic structure with special focus on the influence of three main putative phylogeographic breaks on the population structure of the species (Gibraltar, Oran-Almería, and Peloponnesian), especially on the Atlantic–Mediterranean transition, whose role in determining structuring and evolutionary divergence remains highly discordant across different marine species (reviewed in Patarnello *et al.* 2007). We analysed the same sample collections using the various methods separately and, by comparing different lines of evidence, we reconstructed an integrated view of the patterns of structuring in *L. mormyrus*. The results provide a 'relaxed' interpretive framework, which appears to capture effectively the true complexity of marine population connectivity over space and time.

Materials and methods

Study area and sampling

Sampling locations were chosen to explore spatial genetic structure in Atlanto-Mediterranean *L. mormyrus*, with special focus on the influence of three main putative phylogeographic breaks on the population structure of the species within the Mediterranean Sea and between the Mediterranean Sea and the Atlantic Ocean (Gibraltar, Oran-Almería, and Peloponnesian) (Fig. 1).

An additional sampling site from the Indian Ocean, Durban, South Africa (10 000 km away from the sampling site in the Atlantic Ocean) was also included as an outgroup.

A total of 682 *L. mormyrus* were sampled. Juveniles and adults were collected during the spawning season

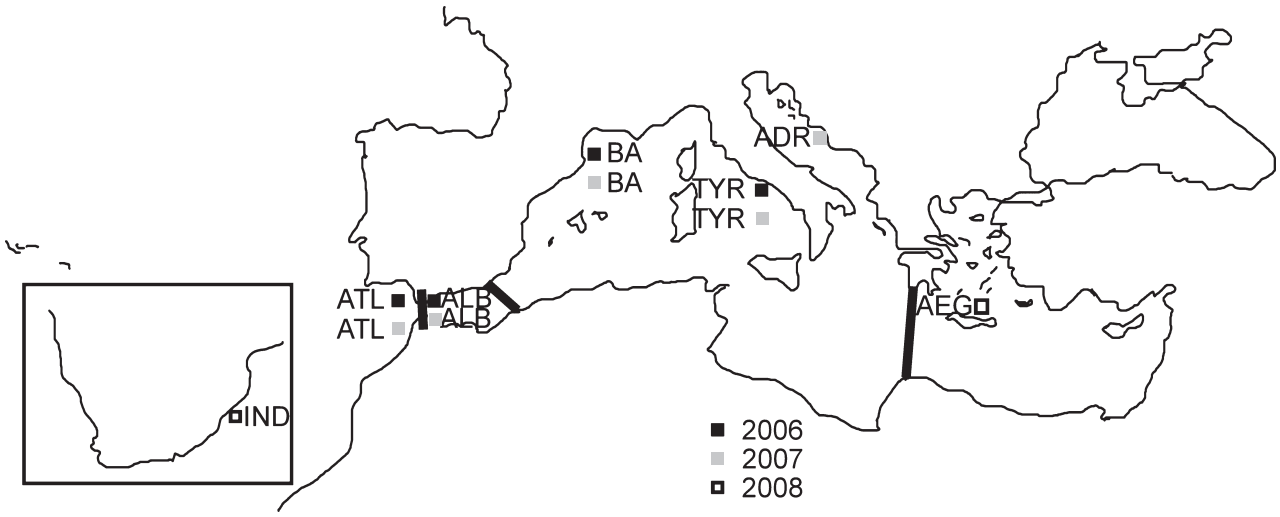

Fig. 1 Map of the sampling area and putative phylogeographic barriers for *Lithognathus mormyrus*. Black lines drawn across sea stretches identify the three putative phylogeographic breaks investigated. Inset box represents South Africa.

between May and September 2006, 2007, and 2008. Four locations were originally sampled in 2006. These were re-sampled in 2007 along with an additional site in the Adriatic Sea. Finally, samples from the Aegean Sea and the Indian Ocean were obtained in 2008.

From each location, adult fish (>15 cm) were collected using trammel and gill nets; juveniles (<15 cm) were collected using hook and line. In total, fin tissue from 662 *L. mormyrus* were obtained and stored in molecular grade ethanol prior to genetic analyses.

Details on the sampling design and on each sampled populations are given in Table 1 and Fig. 1.

Genetic data

DNA was extracted using a slightly modified salt/chloroform extraction protocol (Miller *et al.* 1988), which included an additional chloroform/isoamyl alcohol step after adding the saturated NaCl solution (Petit *et al.* 1999). Polymerase Chain Reaction (PCR) was used to amplify a 263-bp long fragment of the mtDNA control region (CR) gene in 167 individuals, using sparid-specific primers (Ostellari *et al.* 1996). An average of 24 individuals (18–39) were sequenced per site, which is considered adequate for phylogeographic analysis

Table 1 Localities, designations, and genetic variability indices

			GPS coordinates							
	Code	Basins	Latitude	Longitude	*N* (mt/ms/parasites)	H_E	H_O	A_R	*h*	Π
Cádiz 07	ATL 07	Atlantic Ocean	36°33′27.7″	−06°16′00.7″	(22/50/50)	0.8666	0.8666	14.7157	0.602	0.0696
Cádiz 06	ATL 06	Atlantic Ocean	36°33′27.7″	−06°16′00.7″	(−/100/100)	0.8544	0.8420	14.6862	—	—
Málaga 07	ALB 07	Alborán Sea	36°40′48.00″	−4°23′24.00″	(39/100/100)	0.8712	0.8570	15.0032	0.916	0.0182
Málaga 06	ALB 06	Alborán Sea	36°20′14.3″	−005°13′03.7″	(−/22/22)	0.8808	0.8484	16.6666	—	—
L'Estartit 06	BA 07	Balearic Sea	42°02′10.18″	3°12′22.03″	(20/95/95)	0.8370	0.8339	12.0955	—	—
L'Estartit 07	BA 06	Balearic Sea	42°02′10.18″	3°12′22.03″	(−/99/99)	0.8353	0.8397	11.7750	0.889	0.0161
Foce Verde 07	TYR 07	Tyrrhenian Sea	41°24′0.0″	41°24′0.0″	(20/50/60)	0.8308	0.8399	12.4536	0.968	0.0323
Foce Verde 06	TYR 06	Tyrrhenian Sea	41°24′0.0″	41°24′0.0″	(−/40/40)	*0.8291*	0.8277	11.2692	—	—
Duce 07	ADR 07	Adriatic Sea	43°25′48.0″	16°35′24.0″	(20/50/59)	0.8118	0.8333	11.0560	0.895	0.0358
Iraklio 08	AEG 08	Aegean Sea	35°20′38.00″	25°7′11.99″	(18/30/−)	0.8121	0.8144	11.9442	0.980	0.0335
Durban 08	IND 08	Indian Ocean	−29°51′36.0″	31°2′36.0″	(24/26/−)	0.6753	0.6196	10.861	0.967	0.0511

IND, Indian Ocean; ATL, Atlantic Ocean; AEG, Aegean Sea locality; TYR, Tyrrhenian Sea; ALB, Alborán Sea; ADR, Adriatic Sea; BA, Balearic Sea.

Sampling localities with corresponding codes, marine basins and GPS coordinates. Sample sizes (*N*) for mtDNA (mt), microsatellites (ms) and ecological analyses, respectively, are also reported. Diversity indices for microsatellite data (H_E, expected heterozygosity H_O, observed heterozygosity, and A_R, allelic richness) and mtDNA (*h*, haplotype diversity, π, nucleotide diversity) are given in the last five columns.

(Felsenstein 2006). Each 25 μL reaction mixture contained: 2.5 μL 10× INVITROGEN Buffer, 0.2 mM dNTP's, 2 mM $MgCl_2$, 0.4 μM forward primer and 0.4 μM reverse primer, 1 Unit of *Taq* DNA polymerase (Invitrogen, Bio-Sciences Ltd) and 2 ng/μL of template DNA. PCR conditions were as follows: 5 min at 94 °C, followed by 40 cycles of 50 s denaturing at 94 °C, 30 s of annealing at 53 °C and 50 s of extension at 72 °C, with a final 5 min step at 72 °C. PCR products were purified by adding a mix of 1 U of Exonuclease I and 1 U of Shrimp Alkaline Phosphatase (Roche Diagnostics Corporation), in a final volume of 10 μL. Thermocycle conditions for the Exosap purification were as follows: 15 min at 37 °C, and 15 min at 80 °C. Products were sequenced in both directions with the original primers by Macrogen Inc. Chromatograms were edited and assembled in Sequencher 4.2 (Gene codes Corporation); sequences were aligned in MEGA-4 (Tamura *et al.* 2007).

A total of 662 individuals were also PCR amplified and genotyped at nine polymorphic microsatellite loci: Lm68, Lm72, Lm19, Lm86, Lm12, Ad05, Ad66, SaL15 and Sal19 (Brown *et al.* 2005; Franch *et al.* 2006; Sala-Bozano *et al.* 2009). Samples were processed in two multiplex reactions following the protocol in Sala-Bozano *et al.* (2009). Genotyping of individuals was performed by allele sizing on an ABI 3130*xl* Genetic Analyser (Applied Biosystems) using forward primers labelled with NED, PET, FAM and VIC dyes and an internal size standard labelled with LIZ 600 (Applied Biosystems©). Sixty-two individuals (~10%) randomly chosen from the data set were re-extracted and re-amplified for the nine loci. The resulting genotyping error rate was 0.2% per allele, which is considered sufficiently low not to affect studies of population substructure (Bonin *et al.* 2004). The software GeneMapper version4x (Applied Biosystems©) was used to score alleles. The data set was evaluated using MICRO-CHECKER (Van Oosterhout *et al.* 2004) to check for the presence of null alleles, large allele drop out, and possible scoring errors.

Mitochondrial DNA analyses

Nucleotide (π) and haplotype (h) diversity were calculated using DnaSP 4.10.9 (Rozas *et al.* 2003).

ModelTest 3.06 (Posada & Crandall 1998) was used to identify the most appropriate model of evolution for the data set via the Akaike information criteria (AIC) (Burnham & Anderson 1998).

Control region sequences were used to construct a statistical parsimony network in TCS 1.21 (Clement *et al.* 2000) using the default settings. Net Divergence (Da) between the haplogroups identified in the parsimony network was calculated using the program MEGA version 4.0 (Tamura *et al.* 2007). Maximum composite likelihood and a gamma distribution of $\alpha = 0.3158$ (as given by ModelTest) were used and 10 000 bootstrap replicates were employed to calculate the standard error (SE).

Estimates of divergence time (T) among main control region lineages were calculated by the formula $T = Da/2\mu$ where T is given in generations and 2 μ is the mutation rate. We used an evolutionary mutation rate of 11% per million years and assumed a generation time of 4.6 years as seen in Patarnello *et al.* (2007) and Bargelloni *et al.*(2003).

The software Arlequin version 3.11 (Excoffier *et al.* 2005) was employed to estimate variance in haplotype frequencies between samples and basins (F_{ST}-based AMOVA), with significant values tested by 10 000 permutations. Designs of the hierarchical analyses are illustrated in the Results.

Microsatellites analysis

The software LOSITAN (Antao *et al.* 2008), which implements the F_{ST}-outlier detection method (Beaumont & Nichols 1996) was employed to assess whether any of the loci used departed from selective neutrality. The settings were: 95 000 permutations, with a 99% confidence interval.

Population structure was investigated using three complementary approaches: multilocus genotype Bayesian assignment, conventional F_{ST} and the spatially explicit identification of 'barriers' to gene flow.

Individuals were grouped into a most likely number of clusters (K) using the software Structure version 2.1 (Pritchard *et al.* 2000). The software uses a Monte Carlo Markov Chain Bayesian clustering method that maximizes the within-cluster Hardy–Weinberg and linkage equilibrium. All 662 individuals genotyped were used in the assignment. As gene flow can be substantial in marine fish, an admixture model was used. Individuals were assigned to clusters based on the highest probability of membership (Q-statistic). Ten independent runs were performed for each K value (1–6) using 500 000 iterations with a burn-in period of 50 000. The number of clusters was calculated by obtaining the mean posterior probability of the data (log probability of data; $L(K)$) over the 10 independent runs. Individual results from Structure were compared with the results from another Bayesian software, GENELAND (Guillot *et al.* 2005b), which can incorporate geographical information to produce a more accurate inference of population structure and spatial borders between the inferred clusters. The geographical locations were set as the positions where the individuals were sampled. Allele frequencies followed independent Dirichlet distributions. Twenty

independent runs with 200 000 MCMC iterations and a burn-in of 20 000 were conducted. The value of K was set from 1 to 6 clusters. We inferred the number of clusters (K) from the modal value of K for these 20 runs, with the highest posterior probability. Guillot *et al.* (2005a) suggest to infer K in a first run and then to run the algorithm again with K fixed at the previously inferred value in order to estimate the other parameters. Therefore, we ran an additional 200 000 MCMC iterations with K fixed for the value determined by the mode of the posterior distribution of the MCMC chain, and setting the Poisson process equal to the number of sampled individuals.

F_{ST} analysis (Weir & Cockerham 1984) was used to calculate variance in allelic frequencies among locations and samples, and, within samples, between collection years, between sexes, between adults and juveniles and in the case of the Atlantic and the Indian Ocean samples, between individuals belonging to different mitochondrial lineages (see Results). We used GENETIX version 4.5 (Belkhir *et al.* 1996–2004) to estimate pairwise F_{ST} values and test for their significance with 10 000 permutations. This software was also employed to estimate the inbreeding coefficient F_{IS}, for each locus in each site, and hence assess departures from Hardy–Weinberg equilibrium (Wright 1969). Expected and observed heterozygosity, H_E (Nei 1978) and allelic richness (A_R) were also calculated for each site using GENETIX. Sequential Bonferroni corrections were applied for multiple tests (Rice 1989).

To pin down the most significant barriers to gene flow, we used BARRIER 2.2 (Manni *et al.* 2004). This program investigates the possible existence of sharp breaks in genetic differentiation ('barriers') using geographic coordinates and a pairwise F_{ST} matrix. To test for the robustness of the identified barriers, we bootstrapped 100 matrices using a function from R version 2.8.1 (Ihaka & Gentleman 1996; E Petit, UMR CNRS, Paimpont, personal communication). Tests for linkage disequilibrium between all pairs of loci were carried out in Genepop (Raymond & Rousset 1995).

The software Arlequin (Excoffier *et al.* 2005) was used to perform analysis of molecular variance (AMOVA) with the hierarchical designs illustrated in the Results section.

Parasitic infection

Parasitic infection data were obtained by examining specimens from the following localities: Atlantic Ocean, Alborán Sea, Balearic Sea, Tyrrhenian Sea, and Adriatic Sea. Ecto- and endo-parasites of *L. mormyrus* were identified to the lowest possible taxonomic level. Comparisons of infection were carried out using both univariate and multivariate approaches. Kruskal–Wallis nonparametric ANOVA (Systat v.8.0, SPSS) was used to test for geographical variation in each parasite taxon found more than once. Overall parasitic faunas were compared using principal coordinate analysis (PCO) (Gower 1966) with Bray Curtis coefficient as a distance matrix.

A Mantel test was conducted with the software XLSTAT v7.5 (Microsoft Excel) to test for correlation between genetic differentiation (F_{ST}-based matrix) and parasitic faunal variation (Bray Curtis distance matrix) among localities.

Results

Mitochondrial DNA

We identified 80 polymorphic sites out of 263bp across 163 individuals from seven localities, for a total of 100 unique haplotypes (GenBank Accession nos: GQ924784–GQ924883). The overall values for haplotype (h) and nucleotide diversities (π) were 0.916 and 0.0700 respectively. Haplotype diversity (h) ranged from 0.602 to 0.980, with the highest value for the Aegean Sea locality (AEG), followed by the Tyrrhenian Sea (TYR), Indian Ocean (IND), Alborán Sea (ALB), Adriatic Sea (ADR), Balearic Sea (BA) and Atlantic Ocean (ATL) (Table 1). Nucleotide diversity (π) ranged between 0.0161 and 0.0696, with highest values in the Atlantic Ocean and the Indian Ocean localities, followed by ADR, AEG, TYR, ALB and BA (Table 1).

Haplotype 1 was by far the most common; it was found at all sampled locations except for the Indian Ocean. We found four other haplotypes (5, 18, 25, and 26) shared between multiple sites; however, the vast majority of the identified haplotypes were unique to single sites (Fig. 2; Table S1).

The parsimony network analysis (Fig. 2) revealed the existence of three main lineages: Haplogroup I, containing haplotype 1, all the Mediterranean haplotypes and two Atlantic ones (62, 88), Haplogroup II, with five Atlantic haplotypes (63–65, 83–84) and 14 Indian ones (66–76, 78, 82, 99), and finally Haplogroup III, comprising four haplotypes (77, 79, 80–81), all exclusive of the Indian Ocean. The net divergence between Haplogroup I and Haplogroups II and III was 37.8%±17.7 (95% CI: 20.1–55.5%). This gave an estimate for time since divergence between these lineages at about 3.43 Myr (95% CI: 1.83–5.03 Myr). Net divergence between Haplogroups II and III was 16.7±7.1% (95% CI: 9.6–26.3%) with a divergence time between these lineages at about 1.5 Myr ±0.64 (95% CI: 0.86–2.14 Myr).

F_{ST} comparisons among all the localities were calculated (Table 2). The IND locality was found to be significantly different from all other localities. Similar results were found for ATL, which was significantly

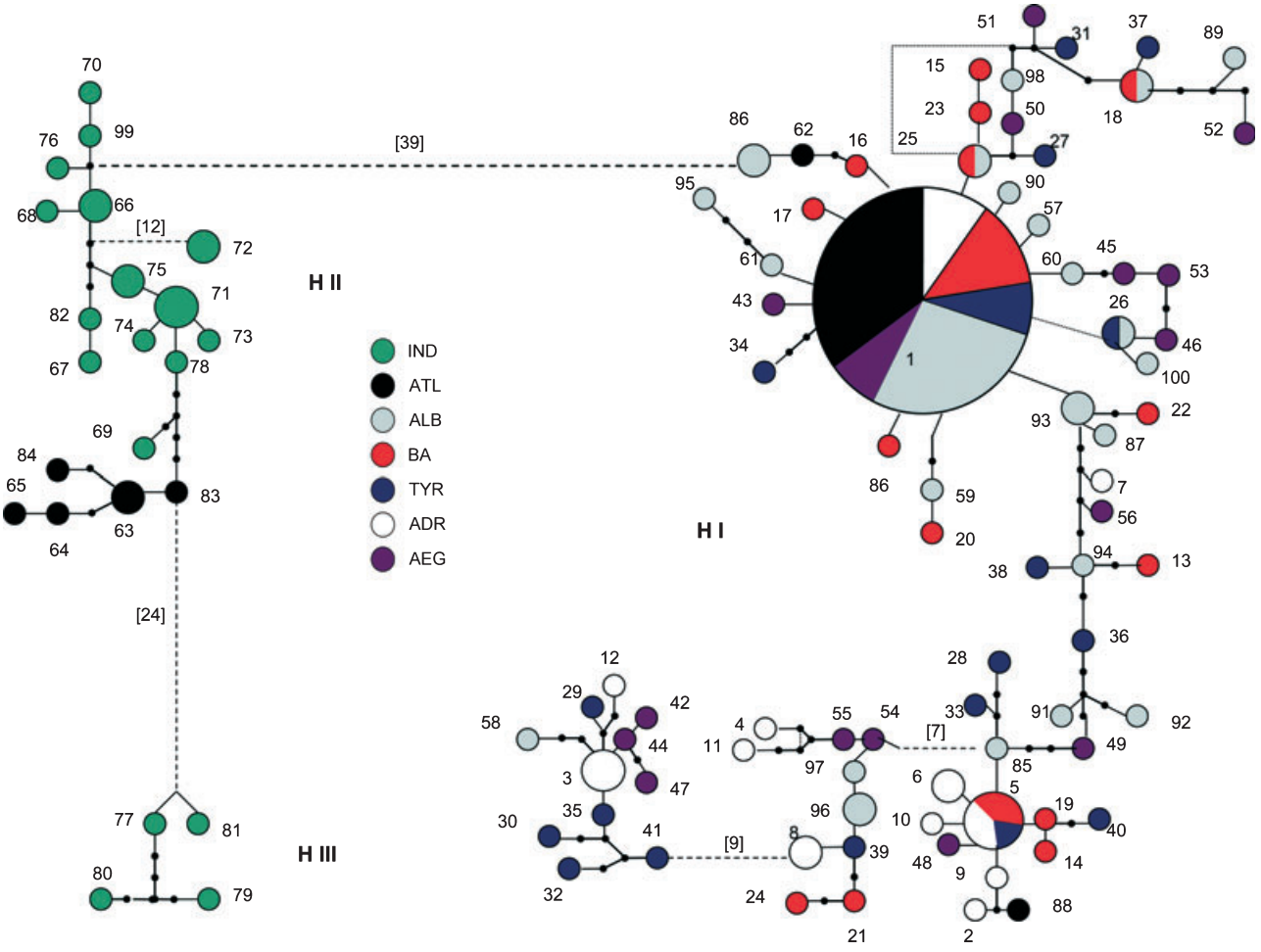

Fig. 2 Network representing the genealogical relationships of the 100 mtDNA control region haplotypes found in the 166 individuals of the striped sea bream sequenced for the study. Circle size is proportional to haplotype frequency. Haplotypes are always one mutational step away from each other except for dashed lines; here numbers in brackets indicate the mutational steps between haplogroups. Dotted lines indicate alternative connections (loops) that could not be resolved using predictions from coalescent theory. Colours refer to geographical locations: green represents Indian Ocean, black Atlantic Ocean, grey Alborán Sea, red Balearic Sea, blue Tyrrhenian Sea, white Adriatic Sea, and purple Aegean. Small black circles represent missing haplotypes.

Table 2 Pairwise F_{ST} values between localities using mtDNA (upper Diagonal) and nuclear DNA, microsatellites (lower diagonal)

	07ATL	06ATL	07ALB	06ALB	07BA	06BA	07TYR	06TYR	07ADR	08AEG	08IND
07ATL			0.0587*	—	0.04196	—	0.10239*	—	0.05613*	0.11968*	0.21675**
06ATL	0.0022			—	—	—	—	—	—	—	—
07ALB	0.0060**	0.0071**			−0.0066	—	0.00192	—	0.00776	0.00961	0.07014**
06ALB	0.0108**	0.0134**	0.0058				—	—	—	—	—
07BA	0.0677**	0.0711**	0.0402**	0.0420**			0.00113	—	0.00135	0.00744	0.07466**
06BA	0.0661**	0.0678**	0.0383**	0.0383**	0.0006				—	—	—
07TYR	0.0644**	0.0686**	0.0382**	0.0387**	0.0048	0.0033			0.00996	−0.00797	0.03577**
06TYR	0.0699**	0.0741**	0.0444**	0.0418**	0.0035	0.0014	0.0044			—	—
07ADR	0.0777**	0.0826**	0.0517**	0.0550**	0.0095**	0.0083**	0.0077*	0.0089*		0.02155	0.08757**
08AEG	0.0861**	0.0864**	0.0562**	0.0527**	0.0257**	0.0244**	0.0270**	0.0317**	0.0331**		0.02992*
08IND	0.1183**	0.1276**	0.1330**	0.1390**	0.2055**	0.2049**	0.2114**	0.2132**	0.2258**	0.2274**	

IND, Indian Ocean; ATL, Atlantic Ocean; AEG, Aegean Sea locality; TYR, Tyrrhenian Sea; ALB, Alborán Sea; ADR, Adriatic Sea; BA, Balearic Sea.

*Significance (initial $\alpha = 0.05$).

**Significance after sequential Bonferroni correction for the mtDNA (initial $\alpha = 0.0023$), for the microsatellites (initial $\alpha = 0.0009$).

different from all other localities with the exception of BA. Differences between the pairwise comparisons for the ATL and IND localities were on the average larger than the ones between ATL and the Mediterranean localities. This can be explained by the fact that within both ATL and IND there are two divergent lineages in sympatry. Consequently, new analyses were performed based on the parsimony network results (Table 5). Genetic relationships among samples are illustrated in the MDS plots in Fig. 3, showing differentiation among samples pooled by geographic areas (Fig. 3A) as well as grouped according to the lineage of origin (Fig. 3B).

The AMOVA recovered significant population structure when we tested for the existence of three groups: one containing the IND samples, a second one for the ATL ones, and a third one for all the Mediterranean ones. $F_{CT} = 0.0717$; $P = 0.049$ (Table 3).

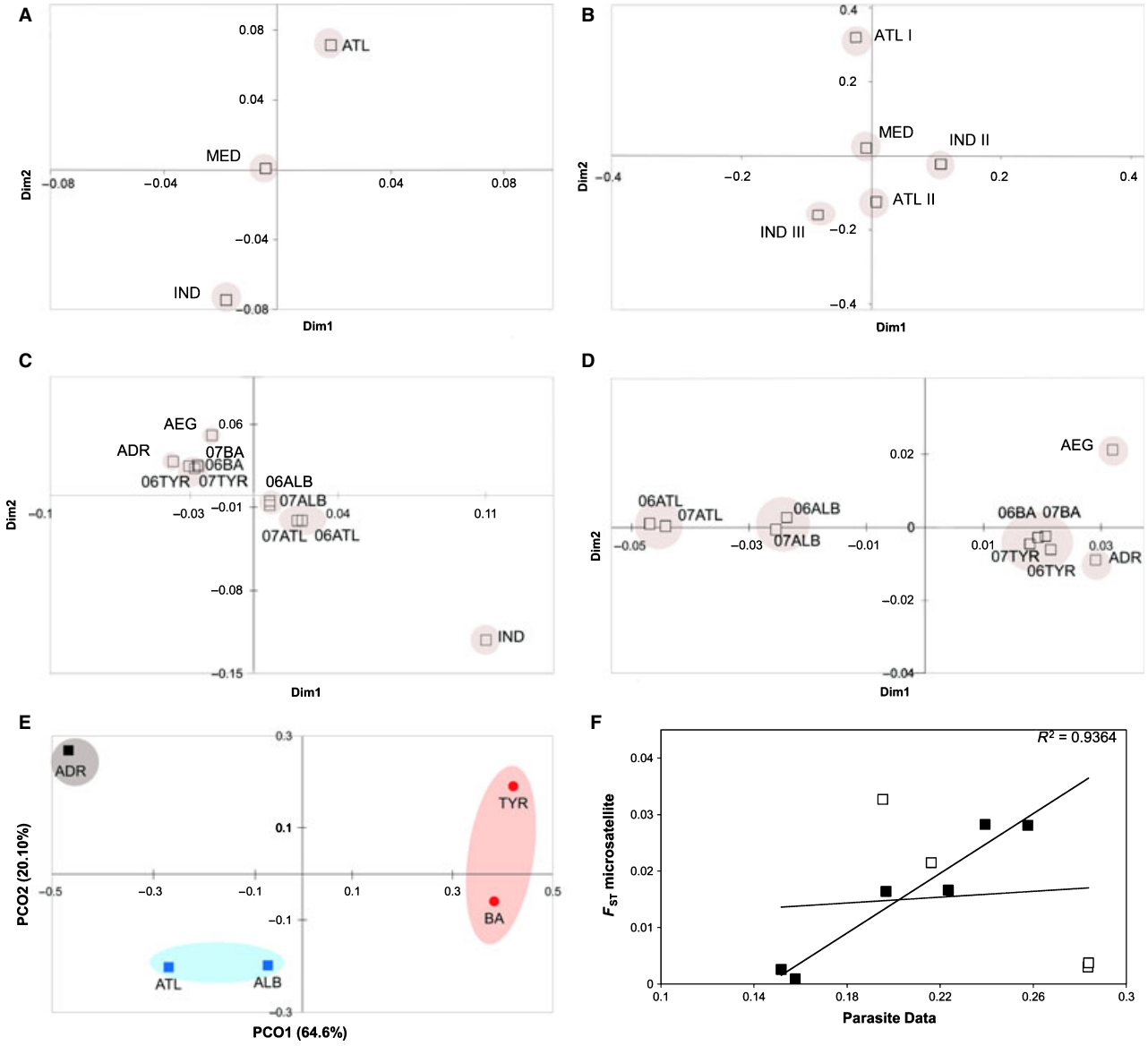

Fig. 3 Genetic and parasite differentiation across geographical areas. (A–B): mtDNA; A includes all populations, B includes all Mediterranean, and the following haplogroups: ATL I-II, IND II-III. (C–D): microsatellites; C includes all populations, in D the Indian Ocean is removed. Each shaded ellipse contains locations that are not significantly different from one another (based on F_{ST} analysis). (E) Principal coordinates (PCO) scatterplot inferred from a matrix of parasite infection. Ellipses group together populations that are not significantly different from one another. (F) Correlation plot between parasitic faunal diversity and microsatellite-based F_{ST} pairwise values. Open squares refer to comparisons involving the Adriatic location; filled squares represent all other comparisons. Continuous trend line shows significant correlation when samples from the Adriatic Sea (open squares) are removed. R^2 represents the coefficient of determination. Dashed line represents trend line for the test including the Adriatic Sea data.

Table 3 Hierarchical analysis of molecular variance (AMOVA) carried out on four different groupings of populations

	Microsatellites		mtDNA	
Comparison	%	*F*-Statistics	%	Φ-Statistics
(A) 1: IND 2: ATL 3: Mediterranean				
Among groups	6.16	0.0616*	7.18	0.0717*
Among populations but within groups	2.02	0.0215*	0.56	0.0060*
(B) 1: IND 2: ATL and ALB 3: Rest Mediterranean				
Among groups	6.9661	0.0696*	3.18	0.0318 NS
Among populations within groups	0.7391	0.0079*	2.16	0.0222*
(C) 1: ATL and ALB 2: BA and TYR 3: ADR and AEG				
Among groups	4.3716	0.0437*		
Among populations within groups	0.6578	0.0068*		
(D) 1: ATL 2: ALB 3: BA and TYR 4: ADR 5: AEG				
Among groups	4.2574	0.0425*		
Among populations within groups	0.2803	0.0029*		

IND, Indian Ocean; ATL, Atlantic Ocean; AEG, Aegean Sea locality; TYR, Tyrrhenian Sea; ALB, Alborán Sea; ADR, Adriatic Sea; BA, Balearic Sea.

Group (A) contains IND, ATL, and all Mediterranean populations pooled together, (B) contains IND, ATL plus ALB, and the remaing Mediterranean locations pooled together, (C) contains ATL plus ALB, BA plus TYR, and ADR plus AEG, (D) contains ATL, ALB, BA plus TYR, ADR, and AEG. Associated with each hierarchical level are: percentage of total variance explained (%), Φ-Statistics for mtDNA and *F*-statistics for nuclear DNA, microsatellites. Estimates of statistical significance are given as: NS, not significant, $*P < 0.05$.

Microsatellites

The F_{ST}-outlier method revealed that locus Lm12 could be under directional selection. All analyses were repeated including and excluding this locus and results did not change; hence we deemed it unnecessary to remove this locus from the analysis. No evidence of null alleles (below 3% across loci and populations), large allele dropout or possible scoring errors was found. Neither did we find evidence of linkage disequilibrium between locus pairs, or deviations from Hardy–Weinberg expectations (Table 1).

Structure revealed a likely number of clusters between 3 and 4. $K = 3$ had a mean value of $-\ln P(D) = -29885.9$ with a standard deviation of 133.6473 whereas $K = 4$ had a mean value of $-\ln P(D) = -29655.5$, but with considerably greater deviation (SD±240.8356). Accordingly, we hypothesized that *L. mormyrus* may be broadly clustered into three or four main demographic groups. Whilst 93.5% of individuals were strongly assigned to either one of two clusters, 75% of the individuals that remained to assign had equal likelihood to belong to either of the two other groups (*Q*-values ≈0.50). Consequently, we opted for a more conservative and statistically supported three-cluster structure as suggested in Pritchard *et al.* (2000). The first cluster contained all the individuals from the Indian Ocean; a second one included individuals from the Atlantic Ocean, two from the Balearic Sea and about 75% of the individuals from the Alborán Sea; the third cluster comprised virtually all individuals from the Mediterranean basin east of the Oran-Almería front, and 16% of the Alborán Sea samples (Fig. 4). A total of 22 individuals had *Q*-values between 0.30 and 0.70 and could not be confidently assigned to either cluster 2 or 3. Twelve out of 22 of these 'mixed genotypes' were collected in the Alborán Sea (Fig. 4).

Estimates from 20 separate MCMC model runs in GENELAND consistently resulted in three clusters largely corresponding to the three described above. All of the South African individuals were also grouped in the first cluster; and respectively 89% and 95% of the individuals grouped in the next two clusters matched with those placed in the Structure clusters 2 and 3.

Overall F_{ST} differentiation was relatively strong and highly significant, with a value of 0.054 ($P << 0.001$), using sample localities as population units, and 0.078 ($P << 0.001$) for the three clusters identified by Structure. Pairwise comparison of F_{ST} values identified six groups among all the samples (Fig. 3C) (Table 2). These comprised: (i) IND, (ii) ATL06-ATL07, (iii) ALB06-ALB07, (iv) BA06-BA07 and TYR06-TYR07, (v) ADR, and (vi) AEG, which were well supported by the relevant AMOVA design ($F_{CT} = 0.0425$, $P << 0.001$; $F_{SC} = 0.0029$, $P < 0.001$) (Table 3). In all cases, adults and juveniles and males and females sampled within each one of the localities in different years were found to be genetically undistinguishable. Fish from the Atlantic and the Indian Ocean locations were also grouped according to the mitochondrial Haplogroup of origin (I

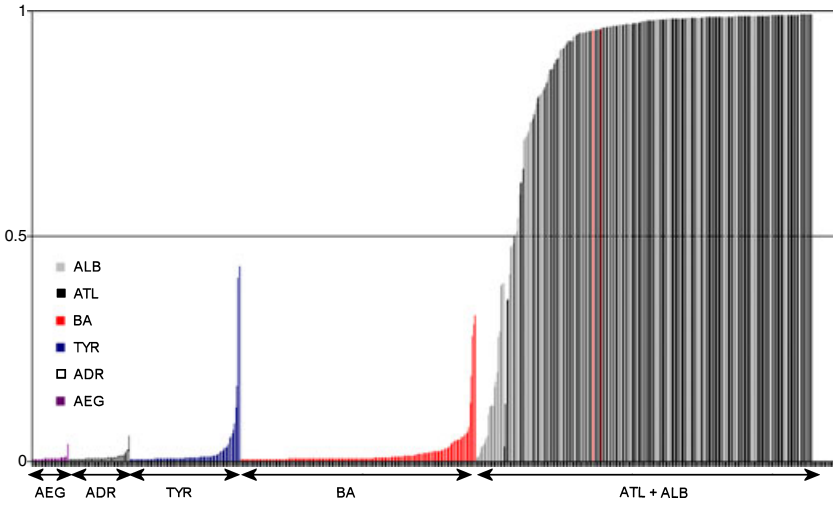

Fig. 4 Individual-based analysis of population structure based on microsatellite data (excluding Indian Ocean samples). Histograms of *Q*-values inferred from Structure. Bars represent the probability of each individual to belong to the Atlantic-Alborán cluster. Colours refer to localities, as in Fig. 2. It can be noticed that the majority of the individuals with *Q*-values between 0.30 and 0.70 are found in the Alborán basin and likely represent recent 'hybrid genotypes' between the two clusters.

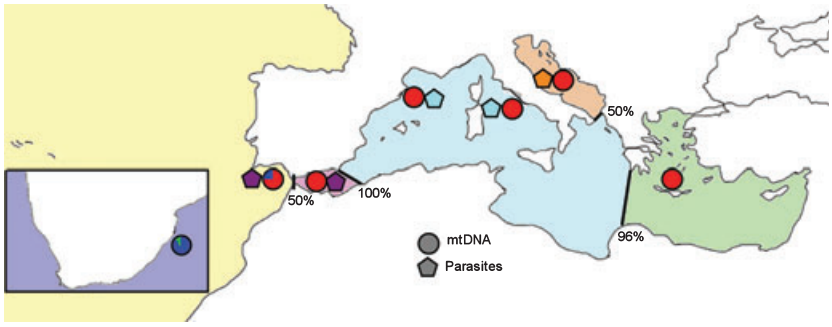

Fig. 5 Synthetic map of geographic population structure of *Lithognathus mormyrus* integrating all different descriptors used. Microsatellite-based geographic structure is represented by differently coloured marine basins, delimited by 'barriers' to gene flow, whose thickness is proportional to their strength, with associated bootstrap values. Circles represent frequency of the three haplogroups identified through mtDNA analysis: red representing Haplogroup I, blue representing Haplogroup II and green representing Haplogroup III. Pentagons refer to parasitic data: different colours represent significantly different parasitic faunas.

and II for ATL; II and III for IND), and proved to be genetically undistinguishable at microsatellites.

We also tested for the significance of putative biogeographical breaks in determining effective barriers to gene flow, using the software BARRIER. The geographically very distant South African population was not included in this analysis. Two barriers had a bootstrap support greater than 95% and two more had a support greater than 50% (Fig. 5). The strongest barrier was in correspondence of the Oran-Almería front, the second one between the western and the eastern Mediterranean basins. Weaker barriers corresponded to the Gibraltar strait and the Adriatic-Ionian boundary respectively (Fig. 5). These results were upheld by alternative AMOVA designs (Table 3) and are consistent with MDS plot visualisation (Fig. 3D), which confirmed that the Alborán individuals were more closely related to the Atlantic ones than to the rest of the Mediterranean populations.

Parasites

Parasite communities did not vary between years within site (not shown). Thirteen different parasitic taxa were identified: nine ectoparasites (three copepods, five isopods, one hirudinean) and four endoparasites (three trematodes and one acanthocephalan). Parasitic infection varied significantly across the study area for most single parasites (Table 4) as well as considering the whole parasitic fauna collectively (Fig. 3E), with western Mediterranean populations clustering separately from ATL and ALB samples, as well as from the Adriatic samples, which clustered separately from all others.

A correlation test between the matrix of microsatellite-based differentiation and a distance matrix inferred from parasitic infection data showed no correlation between data sets (Mantel $Z = 0.49$, $r = 0.06$, $P = 0.039$). However, after plotting the residuals against the regression

Table 4 Kruskal–Wallis results per parasite among the localities and intensity and frequency of parasitic fauna

Locality		*Ceratothoa*	Manca Cymot	Gnathidae	*Caligus*	Hirudinians	*Anilocra*	*Nerocila*	*Pennella*	Trematoda I	Trematoda II	Trematoda III	Acantocephala	Other Copepod
ATL	Frequency	0.00	0.00	0.00	8.00	0.00	3.00	1.00	6.00	6.00	24.00	0.00	0.00	0.00
	Intensity	*0.00*	*0.00*	*0.00*	*0.09*	*0.00*	*0.02*	*0.01*	*0.04*	*0.22*	*0.33*	*0.00*	*0.00*	*0.00*
ALB	Frequency	0.00	0.00	0.00	1.00	1.00	10.00	0.00	3.00	1.00	27.00	3.00	1.00	0.00
	Intensity	*0.00*	*0.00*	*0.00*	*0.01*	*0.03*	*0.11*	*0.00*	*0.02*	*0.01*	*0.56*	*0.02*	*0.01*	*0.00*
BA	Frequency	58.00	2.00	22.00	0.00	0.00	4.00	0.00	0.00	0.00	21.00	7.00	14.00	1.00
	Intensity	*0.55*	*0.02*	*0.42*	*0.00*	*0.00*	*0.03*	*0.00*	*0.00*	*0.00*	*1.20*	*0.08*	*0.25*	*0.01*
TYR	Frequency	61.00	3.00	6.00	0.00	0.00	10.00	0.00	0.00	0.00	15.00	29.00	0.00	0.00
	Intensity	*1.21*	*0.03*	*0.05*	*0.00*	*0.00*	*0.12*	*0.00*	*0.00*	*0.00*	*3.53*	*4.18*	*0.00*	*0.00*
ADR	Frequency	0.00	0.00	0.00	9.00	0.00	3.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00
	Intensity	*0.00*	*0.00*	*0.00*	*0.22*	*0.00*	*0.07*	*0.00*	*0.02*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
H-statistic		207.06	5.50	38.18	46.20	4.12	13.08	3.17	10.84	15.35	17.42	105.39	28.14	2.22
P-value		<0.001	0.23	<0.001	<0.001	0.38	0.01	0.52	0.02	<0.001	<0.001	<0.001	<0.001	0.69

ATL, Atlantic Ocean; TYR, Tyrrhenian Sea; ALB, Alborán Sea; ADR, Adriatic Sea; BA, Balearic Sea.
Frequency represents the amount of individuals infected per population and intensity (reported in italic) is the number of parasites divided for the total number of individuals infected. *H* represents the observed value of the Kruskal–Wallis test statistic and *P* is the associate probability.

predicted values, three out of four of the pairwise comparisons involving the Adriatic were identified as clear outliers. Thus, we performed the analysis again, after the removal of the Adriatic data; in this case a strong association between matrices was detected ($Z = 0.31$, $r = 0.96$, $P < 0.05$; Fig. 3F).

Discussion

The strong spatial patterns identified in this study offer additional support to the tenet that many marine species may exhibit pronounced differentiation, even in the seemingly homogeneous marine environment (Hauser & Carvalho 2008). However, not for the first time (Lemaire *et al.* 2005; Peijnenburg *et al.* 2006; Gonzalez & Zardoya 2007), the use of different markers produced dissimilar and to some extent contrasting scenarios. Although such inconsistencies can potentially complicate the reconstruction of connectivity dynamics in the seascape, we argue that by tailoring the correct interpretation to each one of the descriptors used, it is possible to achieve a deeper understanding of the micro-evolutionary process and consequently resolve population structure and history.

Historical population structure of L. mormyrus

The mtDNA data presented in this study identified three deeply divergent lineages in *L. mormyrus* (Haplogroup I, II, and III in the network analysis of Fig. 2). Haplogroup I (essentially Mediterranean in origin) is separated by 39 mutations from Haplogroup II (Atlantic plus some samples from the Indian Ocean). Bargelloni *et al.* 2003 previously reported 15 mutational steps separating Mediterranean and Atlantic lineages of *L. mormyrus*. This apparent discrepancy might depend on the fact that Bargelloni *et al.* 2003 based their estimates on a much smaller data set (in terms of individuals analysed) and a CR fragment 92 bp shorter than ours; moreover, they employed SSCP analysis, which notoriously does not detect the entire variability of genes.

We estimated that the divergence between these two haplogroups occurred at around 3.4 Ma. Our time estimates are in remarkable agreement with the paleogeography of the Mediterranean–Atlantic area. The Mediterranean Sea is a semi-enclosed Sea with an exclusive connection with the Atlantic Ocean between Cape Trafalgar and Cape Spartel (Gibraltar Strait). It was during the Messinian salinity crisis at about 5.9–5.33 Ma that the Mediterranean became separated from the Atlantic Ocean (Hsü *et al.* 1973). At that time the salinity levels of the Mediterranean Sea increased, while the basin level was lowered down to an almost complete desiccation (Hsü *et al.* 1973). Under these conditions it

is unlikely that pre-Messinian Mediterranean *L. mormyrus* populations survived. During the Pliocene, a tectonic uplifting generated sea level changes and caused the flooding of the Mediterranean Sea with Oceanic water from the Atlantic (Rogl & Steininger 1999). But it was not until the middle Pliocene, at about 3.0 Ma, that the climate conditions improved (Haywood *et al.* 2000), hence allowing the re-colonization of the Mediterranean Sea by *L. mormyrus* and likely generating the separation between lineages. Subsequently, individuals from the Mediterranean Sea must have expanded their range into the Atlantic Ocean. This would explain the coexistence of Haplogroups I and II at the same Atlantic location. Interestingly, no individuals from Haplogroup II were found in the Alborán Sea. This could simply mean that this haplogroup is less common and was not sampled, or that the present day connectivity/gene flow between ALB and ATL is mainly east-to-west with the ALB population being more of a source rather than a sink (Alberto *et al.* 2008).

In the Mediterranean, haplotypes in Haplogroup I are quite evenly distributed across all sub-basins with very little or no geographic structuring. The lack of a clear mtDNA structure within the Mediterranean Sea is further demonstrated by the results of multiple AMOVA tests and conventional F_{ST} analysis (Tables 2–5, and Fig. 3A), which could only detect divergence among the Indian, the Atlantic and the Mediterranean samples.

Coexistence of two highly divergent haplogroups in sympatry is also observed in the Indian Ocean locality, whereby all individuals belong to either Haplogroup II or III. Twenty-four mutational steps separate the two lineages and their divergence likely occurred during the early Pleistocene at about 1.5 Ma. The early Pleistocene eustatic changes in sea levels around the present-day coasts of South Africa (Wigley & Compton 2006) have been often invoked to account for the divergence observed in several marine species in the area (Teske *et al.* 2006). One mechanistic explanation for this evolutionary split may reside in the existence of a deep oceanic trench between the South-eastern African coasts and the Madagascar Ridge, which is believed to have caused comparable lineage divergence in other species (Gopal *et al.* 2006). Our data, however, clearly show that these two lineages came into secondary contact. This finding echoes the recent discovery of highly diverged sympatric lineages in the common octopus (*Octopus vulgaris* L) from the same Durban area (Teske *et al.* 2007), which has been hypothesized to be the result of a recent accidental introduction of a distant population through ballast water. Whilst transportation through ballast water of octopus eggs/juveniles may seem reasonable, it appears highly unrealistic for a benthopelagic coastal and shelf fish such as *L. mormyrus*. Secondary contact in our species may have more likely occurred as a result of rare and irregular – yet not impossible (Gopal *et al.* 2006) – inputs of oceanic waters from East Madagascar to the coast of southern Africa, which could have brought in larvae from the previously separated lineage. However, this hypothesis would have to be specifically tested through a future study focusing on the southern hemisphere portion of the species' distribution range.

Table 5 Pairwise F_{ST} values between groups using mtDNA between geographical groups (upper diagonal) and haplogroups (lower diagonal)

			ATL		IND	
		MED	ATLI	ATLII	INDII	INDIII
MED	MED	—	0.0581**		0.0617**	
ATL	ATLI	0.1545**	—		0.2167**	
	ATLII	0.0752*	0.5206**	—		
IND	INDII	0.0703**	0.3893**	0.0616*	—	
	INDIII	0.0516	0.5523**	0.0366	0.0355	—

IND, Indian Ocean; ATL, Atlantic Ocean.
*Significance (initial $\alpha = 0.05$).
**Significance after sequential Bonferroni correction for the test above (initial $\alpha = 0.016$), for the test below (initial $\alpha = 0.005$).

Present-day population structure of L. mormyrus

Secondary contact of lineages has been extensively documented in a variety of taxonomically unrelated animal groups (Flagstad & Røed 2003; Viñas *et al.* 2004; Noonan & Gaucher 2006; Stefanni & Knutsen 2007). In the present study, however, inference from microsatellite data helped unveiling patterns that go beyond the mere detection of two highly diverged lineages found in sympatry. Bayesian assignment and F_{ST} methods consistently clustered all South African individuals as belonging to a cohesive, panmictic demographic unit, and the same is largely true for the Atlantic individuals, sampled over two consecutive years. Collective mitochondrial and nuclear evidence therefore demonstrates that sympatric individuals carrying highly divergent haplotypes are now interbreeding within a single spawning unit, both in the coastal areas of Cadiz (Atlantic Ocean) and Durban (Indian Ocean).

These findings intriguingly mirror the results of McDevitt *et al.* (2009), who documented the postglacial interbreeding, after secondary contact, of previously diverged caribou (*Rangifer tarandus*) lineages in the Rocky Mountains. In our case, the timescales involved are more staggering, as *L. mormyrus* from both Atlantic and Indian Ocean localities appear to have formed

''hybrid swarms'' from lineages that had been evolving separately for millions of years (using a broadly accepted and relatively conservative evolutionary rate of 11% per million year; Patarnello *et al.* 2007) This necessarily poses new questions regarding the extent, the mechanisms and the significance of intraspecific hybridisation in marine fish.

Lithognathus mormyrus exhibited high levels of genetic structuring (overall microsatellite $F_{ST} = 0.054$, $P << 0.001$), with a spatial pattern that is very consistent with the geography of the Mediterranean basins (Fig. 3D). Excluding the very distant South African population, the strongest barrier to gene flow independently identified by BARRIER and Structure lay inside the Mediterranean basin at the Oran-Almería front, and not at the Gibraltar Strait, as might be expected on the basis of mere geographical considerations. The Oran-Almería front is generated by the anticyclonic gyres produced when the one-way surface current of the Atlantic water flows through the Strait of Gibraltar into the Mediterranean Sea. Such gyre has been shown to determine genetic discontinuities in several species (Naciri *et al.* 1999; Perez-Losada *et al.* 2002; Cimmaruta *et al.* 2005; Alberto *et al.* 2008). Not only did we identify this front as an effective barrier for populations of *L. mormyrus*, but we also detected the existence of a contact zone at the west of this area containing a high frequency of individuals with 'mixed genotypes', which could not be assigned to either the Mediterranean or the Atlantic populations. These individuals likely result from recent admixture events between the two groups east and west of the front. Despite the marked genetic differentiation caused by the Oran-Almería front, it was possible to detect some degree of migrant exchange across the break, which, based on the very coastal and localized habitat use of *L. mormyrus* adults (Suau 1970), is likely to be attained through larval transport.

The second most important barrier to gene flow in the Mediterranean was found to lie between the Ionian and the Aegean Seas (Fig. 5). This barrier corresponds geographically to the quasi-circular anticyclonic front southwest of the Peloponnesus. The same front was previously shown to be associated with genetic breaks in other pan-mediterranean species, such as the anchovy *Engraulis encrasicolus*, the bonito *Sarda sarda* and the cockle *Cerastoderma glaucum (*Pujolar *et al.* 2001; Nikula & Vainola 2003; Kristoffersen & Magoulas 2008). Based on F_{ST} and BARRIER analyses, the Adriatic population was only weakly (yet significantly) differentiated from the Tyrrhenian. Overall, this reinforces the view of a restriction of gene flow between the East and the West Mediterranean, which is consistent with what has been previously reported for several fish species analysed at the scale of the Mediterranean Sea (Borsa *et al.* 1997; Stefanni & Thorley 2003; Béranger *et al.* 2004; Debes *et al.* 2008).

The actual physical break between Atlantic and Mediterranean, in correspondence of Gibraltar (Fig. 5) clearly acts as a weaker barrier to nuclear gene flow than the Oran-Almería front (ATL vs. ALB $F_{ST} \sim 0.01$; Fig. 3D). This is in contrast with the results from mtDNA, which located the break right at the Gibraltar strait. Discrepancies in the levels and patterns of structuring inferred from these two markers have been reported in numerous studies (Lemaire *et al.* 2005; Peijnenburg *et al.* 2006; Gonzalez & Zardoya 2007; Flanders *et al.* 2009), with sex-biased dispersal being accounted for as the main factor in causing such inconsistencies. This seems implausible in our case, given the nature, rather than the extent, of the discrepancies recorded. If a sex-biased dispersal was the explanation for the pattern of differentiation at the Oran-Almería front, this would imply an extensive exchange of female migrants and very strong philopatry for males. This would in turn leave a signature of significant nuclear genetic differentiation between males east and west of the Oran-Almería front (ALB and BA), but not in females. However, this is not the case in our data set, as pairwise comparisons for males and females separately (not shown) provided exactly the same picture. In addition, *L. mormyrus* is a protandrous sequential hermaphrodite, with most reproducing females having already reproduced in previous years also as males. This would entail young philopatric males and greatly dispersing old females. Such an alleged behaviour totally contrasts with the pattern found at the Gibraltar boundary: weak nuclear structure and very strong mtDNA differentiation. Thus, different patterns at nuclear and mtDNA markers are more parsimoniously explained by the different timescales covered by the two markers.

It is worth noting that even the strongest contemporary barrier identified in this study (Oran-Almería) allows for some gene flow between the Balearic and the Alborán Sea, exemplified by the two Balearic fish assigned to cluster 2 and the few Alborán ones assigned to cluster 3 (Fig. 4). Most notably, in the 22 individuals with 'mixed genotypes' – predominantly, but not exclusively found in the Alborán Sea – we find the testimony of the recent interbreeding between cluster 2 and cluster 3. This should not be overlooked; however, we can only speculate on the long-term success of these 'outbred' individuals in the contact zone. The analysis of parasitic faunas demonstrates that populations east and west of the Oran-Almería front exhibit remarkably different infestations. This might make outbred genotypes less adaptable to local ecological interactions at either side of the biogeographical break.

Parasitic fauna

Fish from the same geographical area share local parasites, which tend to be different from parasites infecting fish in another area. This approach is often employed to determine demographically independent fish stocks (MacKenzie & Abaunza 1998). However, parasites cannot be regarded as neutral markers, as their local adaptation results from an arms race between the host defences and the parasite's efficiency to adapt to them (Dybdahl & Storfer 2003). Yet, with some limitations, parasitic faunas can still be employed to inform on demographic connectivity among stocks across ecological time scales (Lester & Mackenzie 2009). Here we conducted a multivariate approach, using ecto- and endo-parasites with very different life histories and transmission modes, arguably increasing the power for discriminating between different host populations.

Parasitic fauna also identified the Oran-Almería front as the main break between Mediterranean and Atlantic. This is in good agreement with microsatellite-inferred structure and a previous study on horse mackerel parasites (MacKenzie *et al.* 2008). However, the Adriatic population – which was only weakly differentiated from the western Mediterranean samples at the genetic level – is characterized by a very divergent parasitic fauna (Fig. 3E). This fauna is paradoxically more similar to that found in the Atlanto-Alborán samples than to the one from the western Mediterranean Sea. This is in contrast with the microsatellite patterns and can only be interpreted in light of the adaptive nature of parasitic infections. We can attempt to explain the similarity between the Atlantic and the Adriatic fauna on the basis of the physico-chemical peculiarities of the Adriatic Sea. This basin is probably the most environmentally divergent of all Mediterranean sub-basins, being characterized by reduced salinity and temperature, and higher primary production (Artegiani *et al.* 1993; Astraldi *et al.* 1999). These conditions have often been likened to a North Atlantic ecological regime (Musco & Giangrande 2005). It is possible that such distinctive environmental features may be responsible for promoting strong adaptation of the Adriatic parasitic fauna, resulting in greater divergence than expected based on geography and differentiation at neutral markers alone. Therefore, the application of parasites as natural tags to examine population structure should be employed with caution and interpreted in a non-neutral conceptual framework.

Implications for evolutionary analysis and conservation

This study highlights the advantages of integrating several methods to gather a more comprehensive understanding of the mechanisms shaping the geographic structure of species. We were able to separate complex historical evolutionary processes from contemporary forces in shaping the structure of *L. mormyrus* populations. Profound climatic and geological transformations determined the isolation and divergence of lineages, which later came into secondary contact and interbred after millions of years of independent evolution. Present-day dynamics produced new, different patterns of structure, demonstrating the effectiveness of oceanographic features in limiting the exchange of migrants between Mediterranean basins (Fig. 5). Relevant to the long-standing debate as to whether Gibraltar or the Oran-Almería front represents the sharpest connectivity break between the Atlantic and the Mediterranean (Patarnello *et al.* 2007), we have shown that the answer may change depending on the descriptor used, as both discontinuities have played and continue to play a significant role as 'semi-permeable' barriers to dispersal at different points in time.

Our results also pinpoint the necessity of taking into consideration that different methodologies will address different points. It is down to the investigators' wisdom to reconstruct the full picture making use of the different streams of information and rescinding from an 'ideal', often unrealistic, expectation of agreement among methods/markers. Invariably, molecular genetic and phenotype-based approaches are most valuable when used in an integrated fashion to identify evolutionary significant, management and/or conservation units (Selkoe *et al.* 2008). Despite the fact that the simultaneous use of these approaches will explore different aspects of biological complexity, there is the need for scientists to convey to managers and policy-makers that the diversity of the available tools is such that good and reliable scientific advice does not necessarily pass through neatly consistent patterns obtained across a suite of markers.

Acknowledgements

This research was funded by the Irish Research Council for Science, Engineering and Technology under the EMBARK Postgraduate Research Scholarship Scheme RS2005120. Additional travel support was provided by the Networking and Training Initiative of the Marine Institute of Ireland. We wish to thank Alberto Arias, Aya Murakami, Mapi Bozano Unzué, Joan Sala Montero and Bill Hutchinson for their invaluable field assistance and Sanja Matic, Bruce Mann, Nota Peristeraki, Club Quatre Jotes, Federación de Pesca Española, and Federazione Italiana Pesca e Attività Subacquee, for providing samples. We would also like to thank Aoife O'Rourke, Ilaria Coscia, Allan McDevitt, Sebastien Puechemaille, and Carlotta Sacchi for technical assistance, and Allan McDevitt, Sebastien Puechemaille, Stephen Palumbi, Kimberly Selkoe and another anonymous reviewer for their valuable suggestions on this paper.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Frequency of haplotypes among populations

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