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In two waters: contemporary evolution of lagoonal and marine white seabream (*Diplodus sargus*) populations

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Abstract

Brackish water ecosystems are often exposed to wide variations in environmental variables, including temperature and salinity, which may cause strong selective pressures on organisms modifying the genetic patterns of species. The aim of this work was to test whether there is a 'divergence-with-gene flow' in coastal lagoon populations of white seabream (*Diplodus sargus*) (Ria Formosa, S Portugal and Mar Menor, SE Spain) respect to four marine populations, by using partial sequences of cyt *b* mitochondrial gene and information from nine microsatellite loci. Genetic diversity was highest in both coastal lagoons (Mar Menor and Ria Formosa) considering mitochondrial and nuclear markers. Although some of F_{ST} population pairwise comparisons were not significant, analyses of molecular variance (AMOVAs) detected differences between groups (coastal lagoon and marine) close to significance. Also, only two haplotypes (Cytb-17 and Cytb-18) were detected in both coastal lagoon sampling sites and these localities (Mar Menor and Ria Formosa) showed the highest number of singletons, some of them with a high number of mutations, as has been already described for other Mar Menor populations (*Pomatochistus marmoratus* and *Holothuria polii*). Also, several tests detected significant positive and balancing selection considering mtDNA and microsatellite data. These data support the hypothesis of selection as one of the drivers of the genetic differences found between coastal lagoon and marine populations. The life strategy adopted by *Diplodus sargus* in coastal lagoons allows it to decrease its mortality rate and improve the heritability of its genes. Also, the increase time spent in coastal lagoons with different temperatures and salinities favours the fitness selection and the maintenance of exclusive haplotypes and genotypes in coastal lagoon inhabitants favouring the 'divergence-with-gene-flow'.

Introduction

Estuaries and coastal lagoons are habitats that display particular characteristics in their nature and exposure to wide variations of the environmental variables, such as temperature and salinity. Coastal lagoons harbour enormous biological productivity and play a pivotal role as nursery areas and feeding grounds for marine estuarine residents and visitors (see Whitfield 1999 and references therein; Mariani 2006). The organisms that survive in these ecosystems are often subjected to strong selective pressures (Cognetti 1978, 1982; Congiu *et al.* 2002; Richards *et al.* 2010; Sanford & Kelly 2011). Fish species that are closely tied to coastal lagoon ecosystems, even if only during a part of their life-cycle, display different levels of genetic structure that result from a complex combination of a number of factors. The most obvious and contrasting factors that generally contribute to the genetic structuring among populations are the tendency for differentiation due to geographic isolation (genetic drift)

and the homogenizing effects of gene flow through dispersal (Ward *et al.* 1994; Francisco *et al.* 2006), but other factors such as divergent selective regimes and population history must also be considered (Beheregaray & Sunnucks 2001; Congiu *et al.* 2002; Guinand *et al.* 2008; González-Wangüemert *et al.* 2009; Iannotta *et al.* 2009). The association of the selective pressures acting in those environments with geographical and eco-physiological discontinuities, can play a relevant role in promoting intraspecific variability and may reflect great evolutionary potential of brackish water environments (Bacci 1954; Iannotta *et al.* 2009; Richards *et al.* 2010; Sanford & Kelly, 2011). Therefore, the analysis of the patterns of geographical distribution of genetic variability of brackish water species may be especially relevant to assess the evolutionary role of these habitats (Congiu *et al.* 2002; Le Rouzic & Carlborg 2007; Angeletti *et al.* 2010). In fact, Beheregaray & Sunnucks (2001) propose that species inhabiting estuarine regions and coastal lagoons may have developed a mechanism of 'divergence-with-gene flow' through local adaptation. The relevance of this approach may aid the early detection of species formation (incipient species) in estuaries when they are able to breed into brackish systems.

The white seabream *Diplodus sargus* (Linnaeus, 1758) is a commercial fish species which is distributed in the Atlantic and Indian Oceans, the Mediterranean Sea and the Persian Gulf. The life history of the white seabream shows a pattern consistent with digynic hermaphroditism achieving sexual maturity during the second or third year of life. Spawning occurs in the open sea from March to June and the onset and duration of spawning season in the seabream appears to be influenced by seawater temperatures (Morato *et al.* 2003). *Diplodus sargus* are non-guarders and have pelagic eggs and larvae which can spend up to 4 weeks in the open sea before reaching favourable sites for recruitment (González-Wangüemert *et al.* 2004a). This species behaves as 'cyclic migrants', migrating into lagoons after metamorphosis and spending the early stages of their life cycle in these environments. Juveniles recruit to shallow areas (<5 m), whereas adults are more abundant in the surf zone (Pajuelo & Lorenzo 2004) in depths between 10 and 50 m depending upon substrate availability (Harmelin-Vivien *et al.* 1995). The adults are present in a diverse variety of habitats, including coastal rocky reefs, sandy bottoms and seagrass beds (Domingues *et al.* 2007).

The aim of this work was to test whether there is a 'divergence-with-gene flow' in coastal lagoon populations (Ria Formosa, S Portugal and Mar Menor, SE Spain) of white seabream using partial sequences of cyt *b* mitochondrial gene and information from nine microsatellite loci.

Material and Methods

Sampling sites

In the present study, six sites representative of both marine and coastal lagoon environments were sampled (Fig. 1). Marine samples were obtained from two Atlantic sites located in Northern Spain and Southern Portugal (A Coruña, GL; Quarteira, QR respectively) and from two western Mediterranean locations in France and Southeastern Spain (Banyuls, BY; Murcia, MU). Coastal lagoon samples were collected from two partially enclosed coastal bodies: Ria Formosa (FM) in South Portugal and Mar Menor (MM) in Southeastern Spain (Fig. 1).

The spatial scales chosen are similar for the Atlantic and Mediterranean: one marine sample near each coastal lagoon about 20 km (Quarteira-Ria Formosa and Murcia-Mar Menor) and one marine sample far from coastal lagoon about 850 km (Galicia-Ria Formosa and Banyuls-Mar Menor).

The Ria Formosa is a large tidal lagoon extending for about 55 km along the south coast of Portugal (36°58′ N, 8°02′ W to 37°03′ N, 7°32′ W), with a maximum width of 6 km (Fig. 1). A seaward belt of dunes protects a system of salt marshes, subtidal channels and tidal flats, with a total surface area of approximately 170 km^2. A strongly branched system of creeks and channels is connected with the ocean by six outlets, with an average depth of <3 m. It has a water volume generally well mixed vertically, although the inner parts of the lagoon are poorly mixed and occasionally have eutrophication problems (Newton & Mudge 2003). The system has semi-diurnal tides, with 50–75% of the water volume exchanged during each tide (Aguas 1986; Ribeiro *et al.* 2008). The western part of the lagoon does not receive any permanent freshwater input: salinity is 35.5–36.9 PSU all year, except for surface waters for brief periods after heavy winter rainfalls (Falcao *et al.* 1992). Water temperature varies from 12 to 28 °C (Sprung 1994).

The Mar Menor is a hypersaline coastal lagoon with a surface area of about 135 km^2, located in a semi-arid region of the SW Mediterranean, SE Spain (Fig. 1). It is isolated from the Mediterranean Sea by a 22-km-long sandy bar (La Manga), crossed by five very shallow channels. In the early 1970s, one of these channels (El Estacio) was dredged and widened to make it navigable. The mean depth is 3.5 m with a maximum depth of 6 m. The salinity of the lagoon waters ranges between 39 and 47 psu, due to high evaporation and low exchange rates with the Mediterranean Sea. Water temperature ranges from 10 °C in winter to 31 °C in summer. There are more than 20 cataclinal watercourses in the watershed, most of them discharging into the southern basin of the lagoon (Pérez-Ruzafa *et al.* 2005a). Three main basins have been

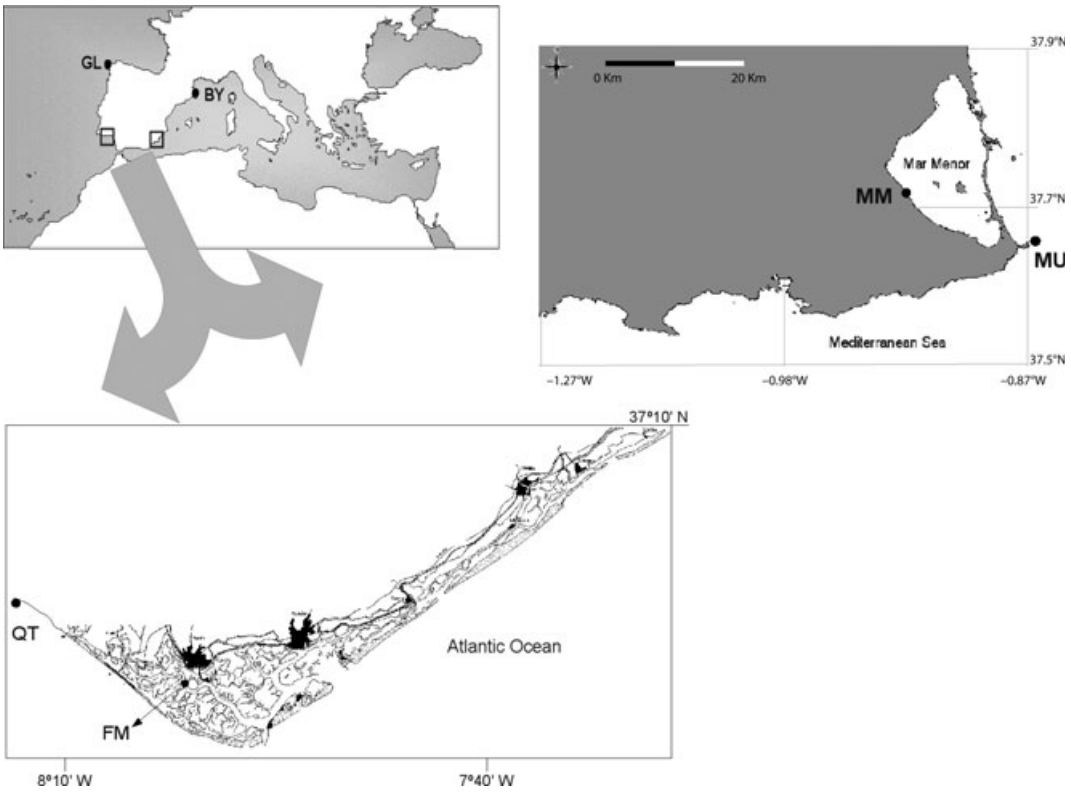

Fig. 1. Location of the sampling sites. BY, Banyuls; MM, Mar Menor, MU, Murcia; RF, Ria Formosa; QR, Quarteira; GL, Galicia.

differentiated inside the lagoon on the basis of their hydrographical characteristics (Pérez-Ruzafa *et al.* 2005b; González-Wangüemert *et al.* 2009).

DNA extraction, PCR, sequencing and genotyping

We analysed a total of 259 individuals of *D. sargus* from samples collected mostly at the local fish markets and a few collected by SCUBA diving during 2006–2007 (259 individuals using microsatellites and 142 individuals using cyt *b*). Muscle samples were removed from fresh fish and stored in 100% ethanol immediately after collection. Total genomic DNA was extracted from small (3–5 mg) sections of tissue following the protocol based on Sambrook *et al.* (1989) with some modifications (González-Wangüemert *et al.* 2011). The extracted DNA was resuspended in elution buffer and stored at −20 °C until further utilization.

The mitochondrial cyt *b* region (661 bp) was amplified using the primers designed in adjacent regions coding for the transfer tRNA (tRNA) glutamate, cbtd2 (5′-AATGAYWTGAAAAACCACCGTTG-3′) and tRNA tryptophan, cbtr2 (5′-CGGMTTACAAGRCCGRYGCT-3′) described by Jousson *et al.* (2000). For each PCR, a 24-μl total volume contained 2.5 μl of 10× buffer (Ecogen), 1.5 mM of $MgCl_2$, (Ecogen), 200 μM dNTP mix, 0.5 U *Taq* DNA polymerase (Ecogen), 0.25 μM of each primer; and 5–50 ng DNA.

PCR cycles were performed on a Biometra T3 Thermocycler under the following conditions: 3 min at 94 °C, followed by 40 cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 2 min; finishing with an extension step at 72 °C for 5 min. PCR products were electrophoresed and purified using the ExoSAP-IT (USBEurope GmbH). Purified DNA was sequenced in one direction with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing v. 2.0-ABI PRISM; Applied Biosystems) and then analysed with an ABI 3700 automated sequencer. Chromatograms obtained from the automated sequencer were read using the BIOEDIT sequence alignment editor (Hall 1999).

Samples were also screened for variation at each of eight microsatellite loci previously isolated and characterized for *Diplodus vulgaris* by Roques *et al.* (2007) and one microsatellite (SAI19) for *Sparus aurata* by Brown *et al.* (2005) (Table 2). Polymorphisms of eight microsatellite loci (Roques *et al.* 2007) were tested by multiplex PCR performed in 20 μl total volume, which include 50 ng of DNA, 2 mM $MgCl_2$, 0.25 μM of each primer, 200 μM dNTPs, buffer and 0.5 U *Taq* polymerase. Reaction conditions were as follows: an initial denaturation step of

5 min at 95 °C, eight cycles consisting of 45 s at 92 °C, 45 s at 53 °C annealing temperature, 45 s at 72 °C followed by an additional 24 cycles consisting of 30 s at 92 °C, 30 s at 55 °C annealing temperature, and 30 s at 72 °C. Locus SAI19 was amplified in 10 μl total volume, including 40 ng genomic DNA, 0.75 μM forward and reverse primers, 130 μM dNTPs, 1.5 mM $MgCl_2$, buffer and 0.5 U *Taq* polymerase. The PCR reactions were performed with the following programme: initial denaturation step of 95 °C for 3 min, followed by 30 cycles of 50 s at 95 °C, 50 s at 60 °C and 1 min at 72 °C, then a final extension step of 30 min at 60 °C.

Individuals were genotyped by assessing allele size on an ABI 3700 automated sequencer, using forward primers labelled with FAM (Sigma), HEX (Sigma) and NED (Applied Biosystems). Allele scoring was carried out using GENOTYPER software (Applied Biosystems).

Genetic analysis: mtDNA

Haplotype and nucleotide diversity values were calculated using ARLEQUIN version 3.11 (Excoffier *et al.* 2005). The same program was used to calculate the pairwise genetic distances (F_{ST}) and their significance by performing 10,000 permutations. We also performed an AMOVA to examine hierarchical population structure. We considered a lagoon group (Mar Menor and Ria Formosa) and a marine group (Banyuls, Murcia, Quarteira, Galicia), executing 16,000 permutations to guarantee having <1% difference with the exact probability in 99% of cases (Guo & Thompson 1992).

Intraspecific relationships were established by means of phylogenetic networks using a statistical parsimony method (Templeton *et al.* 1992) implemented in the software package TCS, version 1.13 (program available at http://darwin.uvigo.es/software/tcs.html) (Clement *et al.* 2000).

This method, based on population genetics theory, determines a 95% statistical confidence limit for the maximum number of nucleotide sites expected to differ between two given haplotypes without any superimposed substitutions, the '95% confidence limit of parsimony' (Templeton *et al.* 1992). This method is preferred to others as it displays higher resolution in cases where the level of divergence among sequences is low (Posada & Crandall 2001), as in the case of intraspecific derived sequences.

Genetic analysis: microsatellites

Allele frequencies, allelic richness, mean allelic richness, number of exclusive alleles, expected (H_e) and observed (H_O) heterozygosity were calculated using GENETIX v.4.1 software (Belkhir *et al.* 1996–2004). Linkage disequilibrium was tested for each locus–population combination using GENEPOP v.3.1 (Raymond & Rousset 1995), which employed a Markov chain method with 10,000 iterations, following the algorithm of Guo & Thompson (1992). Deviations from Hardy–Weinberg genotype proportions were characterized by F_{IS} and tested using exact tests in the GENEPOP software. In instances where the observed genotype frequencies deviated significantly from HWE, the program MICRO-CHECKER v.2.2.3 (Van Oosterhout *et al.* 2004) was used to infer the most probable causes of such HWE departures.

Differentiation among locations was quantified by F_{ST} (using the estimator θ of Weir & Cockerham 1984) and tested for allele-frequency heterogeneity using an exact test. The null hypothesis of no genetic differentiation between locations was tested by permutating individuals, using the GENETIX software.

A hierarchical AMOVA was carried out to assess the component of genetic diversity attributable to (i) variance between groups, (ii) variance among locations within groups, and (iii) variance within locations. The ARLEQUIN program was used to carry out all these analyses.

Previous work using mtDNA markers have demonstrated changes in *Diplodus sargus* population sizes (Domingues *et al.* 2007; González-Wangüemert *et al.* 2010, 2011), therefore we tested the existence of bottlenecks in all locations. Bottlenecks can be detected by the depletion of allele numbers and heterozygosity excess. To determine whether a population exhibits a significant number of loci with heterozygosity excess, we used the *Sign* and *Wilcoxon* tests implemented in the program BOTTLENECK v.1.2.2 (Piry *et al.* 1999). Computations were based on both stepwise mutation (SMM) and two-phase mutation (TPM) models (Di Rienzo *et al.*1994).

Correlation of mtDNA and microsatellites with geographical distance

We tested for the presence of correlation between geographic and genetic distance measured by F_{ST} for all pairs of populations by performing a Mantel's test (Mantel 1967) using the ARLEQUIN software (Schneider *et al.* 2000). Statistical significance of the values was obtained via 9999 random permutations (Estoup & Angers 1998). Geographic distance between locations was estimated in kilometres as the most direct aquatic route between sites.

Selection

ARLEQUIN software was used to test for departures from mutation-drift equilibrium with Tajima's D-test (Tajima 1989) and Fu's F_S (Fu 1997). The significance of these statistics was tested by generating random samples under the hypothesis of selective neutrality and

population equilibrium, using a coalescent simulation algorithm adapted from Hudson (1990).

In addition, the number of synonymous substitutions per synonymous site (dS) and the number of nonsynonymous substitutions per nonsynonymous site (dN) were estimated using the Z-test implemented in MEGA v. 4.0 (Tamura *et al.* 2007) according to Nei & Gojobori (1986) with the correction of Jukes & Cantor (1969) for multiple substitutions. The variances of dS and dN were computed by bootstrap (10,000 replicates). With this information, the null hypothesis of neutral evolution (H0: dN = dS) *versus* the hypothesis of positive selection (H1: dN > dS) was tested using a Z-test: $Z = [dN - dS)/\sqrt{(Var(dS) + Var(dN)]}$.

The maximum-likelihood method (Yang *et al.* 2000) implemented in the program HyPhy of the MEGA 5.05 software package was used to test whether codon sites on the *cytb* gene were affected by positive selection. The models were Tamura & Nei (1993) and Felsenstein (1981). To provide phylogenetic information for the analysis, the best tree for cyt *b* sequences was chosen using the neighbour-joining method (MEGA v 5.05).

A selection detection workbench Lositan (Antao *et al.* 2008) based on the FDIST FST outlier methods of Beaumont & Nichols (1996) was also used to evaluate the neutrality of the microsatellites. For all runs 50,000 simulations were generated with 'neutral mean FST' and 'force mean FST', to increase the reliability of the mean FST.

Results

mtDNA haplotypic variation

A 661-bp portion of the cyt *b* mtDNA was sequenced and confidently aligned for 142 individuals. In total, 35 segregating sites were noticed across all individuals shown in coastal lagoon samples (Ria Formosa and Mar Menor), the highest number of non-synonymous segregating sites (Table 1). Twenty-five different haplotypes were found and their sequences were registered in GenBank (EF421850–EF421854, EF421841, EF421844, EF421845, EF421832–EF421837, JN380908–JN380918).

Haplotypic diversity differed among samples (0.551–0.853), with the coastal lagoons showing the highest values (FM: 0.757; MM: 0.853) and the nucleotide diversity values ranging from 0.001 (QR) to 0.005 (FM) (Table 1). Mar Menor and Ria Formosa localities had the highest number of haplotypes (9 and 12, respectively) and exclusive haplotypes (4 and 7, respectively).

Microsatellite variability, Hardy–Weinberg equilibrium

Microsatellite loci displayed high levels of allele diversity (3–27 alleles) and observed heterozygosity (0.103–0.880) (Table 2). All populations showed similar variability in heterozygosity. Both coastal lagoon samples showed a high number of exclusive alleles (MM: 9; FM:8). Ria Formosa had the highest number of alleles (FM: 126), while Mar Menor lagoon had 117.

Linkage disequilibrium was not observed, although some loci showed single significant values for some locations: Dvul11/Dvul184, Dvul12/Dvul6, Dvul38/Dvul6, Dvul4/Dvul61 and SaI19/Dvul38, Dvul61, Dvul6.

Significant departures from Hardy–Weinberg equilibrium were observed in six localities. When all loci were analysed separately, the departure was mainly due to three loci (Dvul61, Dvul6 and Dvul4). The MICRO-CHECKER software detected the presence of null alleles in Dvul6 and Dvul61 loci for all locations and in Dvul4 locus for three locations (Banyuls, Quarteira, Murcia). In spite of null alleles, we did not find an overestimation of F_{ST} values; in fact, some values were higher when we eliminated the microsatellite loci with null alleles. Therefore we kept all loci for subsequent analysis.

Genetic divergence among localities

Some of the F_{ST} population pairwise comparisons were significant ($P < 0.05$) for mitochondrial data (Table 3). Banyuls showed high and significant F_{ST} values between all samples except for Galicia ($F_{ST} = 0.04$; $P > 0.05$). Ria Formosa also presented high values between Banyuls, Murcia and Quarteira despite the geographic proximity

Table 1. Genetic diversity measures for the populations of *Diplodus sargus* studied using cyt *b*.

populations	habitats	N	HN	EHN	Sn	Ss	π	h
MM	Coastal lagoon	22	9	4	8	0	0.0021	0.8528
FM	Coastal lagoon	24	12	7	21	2	0.0045	0.7572
BY	Marine	22	6	2	6	0	0.0014	0.5513
MU	Marine	25	7	2	6	0	0.0016	0.5604
GL	Marine	24	6	1	5	0	0.0011	0.5507
QR	Marine	25	4	1	3	0	0.0009	0.5524

N, number of individuals; HN, number of haplotypes; EHN, exclusive haplotype number; Sn, number of nonsynonymous segregating sites; Ss, number of synonymous segregating sites; π, nucleotide diversity; h, haplotype diversity.

Table 2. Levels of genetic variation observed at nine microsatellite loci.

		coastal lagoon		marine			
		MM	FM	BY	MU	GL	QR
locus	size sample	40	50	40	45	44	40
DVUL11	Allele number	7	10	4	7	4	5
	Exclusive alleles number	0	3	0	0	0	0
	H_O	0.450	0.700	0.667	0.328	0.520	0.333
	H_e	0.520	0.646	0.582	0.293	0.422	0.381
DVUL12	Allele number	5	8	3	5	4	6
	Exclusive allele number	0	1	0	0	0	0
	H_O	0.125	0.620	0.333	0.310	0.280	0.389
	H_e	0.571	0.758	0.549	0.342	0.364	0.520
DVUL38	Allele number	9	10	4	7	5	5
	Exclusive allele number	2	1	0	0	0	0
	H_O	0.447	0.480	0.444	0.491	0.480	0.278
	H_e	0.695	0.667	0.437	0.626	0.556	0.536
DVUL4	Allele number	20	19	13	19	20	11
	Exclusive allele number	3	0	0	3	1	1
	H_O	0.750	0.760	0.563	0.632	0.840	0.500
	H_e	0.888	0.918	0.852	0.889	0.920	0.863
DVUL84	Allele number	12	8	7	8	8	9
	Exclusive allele number	4	0	0	0	0	1
	H_O	0.775	0.820	0.778	0.741	0.840	0.778
	H_e	0.843	0.831	0.799	0.796	0.802	0.815
SAI19	Allele number	4	7	5	4	3	4
	Exclusive allele number	0	1	0	0	0	2
	H_O	0.103	0.300	0.167	0.103	0.400	0.111
	H_e	0.168	0.383	0.253	0.099	0.374	0.250
DVUL33	Allele number	13	15	9	16	11	9
	Exclusive allele number	0	1	0	4	0	1
	H_O	0.750	0.880	0.722	0.862	0.880	0.389
	H_e	0.835	0.878	0.833	0.841	0.869	0.775
DVUL61	Allele number	27	23	18	25	18	16
	Exclusive allele number	0	0	0	0	0	0
	H_O	0.539	0.510	0.500	0.418	0.440	0.500
	H_e	0.937	0.921	0.926	0.922	0.905	0.909
DVUL6	Allele number	20	26	17	27	16	11
	Exclusive allele number	0	1	0	1	0	0
	H_O	0.351	0.413	0.333	0.429	0.280	0.389
	H_e	0.919	0.932	0.929	0.946	0.918	0.830
all LOCI	Mean allele number	13	14	8.889	13.111	9.889	8.444
	Allele number	117	126	80	118	89	76
	Exclusive allele number	9	8	0	8	1	5
	Mean H_O	0.477	0.609	0.501	0.479	0.551	0.407
	Mean H_e	0.708	0.770	0.684	0.639	0.681	0.653

MM, Mar Menor; FM, Ria Formosa; BY, Banyuls; MU, Murcia; GL, Galicia; QR, Quarteira; H_O, observed heretozygosity; H_e, expected heterozygosity.

to this last locality (F_{ST} = 0.06; F_{ST} = 0.05; F_{ST} = 0.04, respectively). Mar Menor coastal lagoon only showed significant genetic differences with Banyuls and Quarteira (F_{ST} = 0.11; F_{ST} = 0.07, respectively). F_{ST} values obtained from microsatellite data were lower than those observed from mitochondrial data, although the majority were significant (Table 3). Ria Formosa and Mar Menor coastal lagoons showed significant differences between all localities except for Banyuls. Murcia and Quarteira presented significant F_{ST} values between all samples.

The AMOVA test comparing coastal lagoon and marine groups (cyt *b* data) showed differences that were close to significance (3.9%; F_{CT} = 0.027; P = 0.06), and the rest of variance was distributed among populations within each group and within populations (Table 4). Using microsatellite data and the same groups, the AMOVA

Table 3. Pairwise fixation indices between six *Diplodus sargus* populations based on mtDNA cyt *b* region haplotypes (F_{ST}, below diagonal) and nine microsatellite loci (F_{ST}, above diagonal).

	BY	QR	FM	GL	MM	MU
BY	–	0.014*	0.007	0.009	0.004	0.013*
QR	0.063**	–	0.030*	0.024*	0.029*	0.024*
FM	0.064**	0.040*	–	0.017*	0.018*	0.030*
GL	0.040	−0.020	0.027	–	0.023*	0.014*
MM	0.109***	0.065*	0.012	0.038	–	0.018*
MU	0.095***	0.033	0.049***	0.033	0.026	–

*P < 0.05, **P < 0.01,***P < 0.001.

indicated that the highest proportion of the total variance was attributed to differences within locations and only 0.49% of the variation was attributed to marine and lagoon groups (Table 4).

No pattern of genetic differentiation (F_{ST}) in relation to geographic distance was observed using cyt *b* data (Z = 2250.68.23; G = −1.190; Pearson coefficient, r = −0.323; P = 0.101) or microsatellite data (Z = 2196; G = −1.193; Pearson coefficient, r = −0.324; P = 0.173), showing no evidence of increasing genetic differentiation with geographic distance.

Neutrality, selection and bottlenecks

Using Tajima's D test, a significant deviation from the neutral model was detected only in Ria Formosa (Tajima's D = −1.901; P = 0.017); however, Fu's F_S test was significant in five locations (Ria Formosa, Mar Menor, Murcia, Banyuls and Galicia).

The null hypothesis of evolution according to the neutral model could be rejected with a general Z-test for all samples. The dN-values were significantly larger than the dS-values for the Z-tests with all samples (HA: dN > dS; Z = 2.468; P = 0.007). Also, the maximum-likelihood method using HyPhyl showed that 26 sites (AA10, 68, 72, 75, 89, 91, 104, 106, 119, 128, 130, 134, 145, 148, 153, 167, 168, 169, 170, 172, 173, 182, 183, 187, 190 and 194) of the cyt *b* gene were under positive selection.

Coalescent simulations (performed by LOSITAN software), using overall FST (FST = 0.019) as an expected value for neutral markers and infinite allele model, found the upper and lower FST limits at the 0.95 probability level (Fig. 2). Among the nine polymorphic loci, Dvul12, Dvul6, Dvul84 and Dvul4 appeared to be candidates for selection (Fig. 2). Dvul12 and Dvul6 loci were candidates for positive directional selection (H_e = 0.5601, FST = 0.0597, P = 0.9933; and H_e = 0.9607, FST = 0.0327, P = 0.9983 respectively), whereas Dvul4 and Dvul84 (H_e = 0.9139, FST = 0.0100, P = 0.0098; and H_e = 0.8338, FST = 0.0056, P = 0.0087, respectively) were considered to be balancing selection candidates.

We found an indication of recent reductions in effective population size in Mar Menor coastal lagoon (Sign test: TPM, P = 0.026, SMM, P = 0.0003; Wilcoxon test: TPM, P = 0.04, SMM, P = 0.001). Banyuls and Galicia did not show any evidence of bottlenecks, and Ria Formosa showed significant values only for the tests based on SMM (Sign test: SMM, P = 0.028; Wilcoxon test: SMM, P = 0.037). Quarteira and Murcia showed significant values for the Sign and Wilcoxon tests based on SMM but these values were not significant when we used the TPM model (P > 0.05).

Haplotype network

The network can be characterized as a star phylogeny with low phylogeographic structure (Fig. 3). The most frequent cyt *b* haplotype (Cytb-1), represented by the biggest circle, included individuals from all localities, such as the second and third most common haplotypes (Cytb-5 and Cytb-6). Seven singletons (Cytb-12, Cytb-13, Cytb-14, Cytb-15, Cytb-16, Cytb-19 and Cytb-20) were found in Ria Formosa and four (Cytb-21, Cytb-22, Cytb-23 and Cytb-24) in Mar Menor. Also, only two haplotypes (Cytb-17 and Cytb-18) were detected in both coastal lagoon samples (FM and MM).

The star phylogeny allows a tentative assignment of network polarity working outwards from the presumed most ancient types in the interior (Crandall & Templeton 1993). This means that Cytb-1 is the most ancient

Table 4. Analysis of molecular variance (AMOVA) among *Diplodus sargus* mtDNA cyt *b* region haplotypes and microsatellites based on F_{ST}.

molecular marker	source of variation	total variance (%)	fixation indices	P
cyt *b*	Among groups (lagoon-marine)	3.9	F_{CT} = 0.027	0.060
	Among populations within groups	**2.35**	**F_{SC} = 0.016**	**0.017**
	Within populations	**93.75**	**F_{ST} = 0.646**	**0.001**
microsatellites	Among groups (lagoon-marine)	0.49	F_{CT} = 0.005	0.073
	Among populations within groups	**2.09**	**F_{SC} = 0.021**	**0.000**
	Within populations	**97.41**	**F_{ST} = 0.026**	**0.000**

Significant values (P < 0.05) in bold.

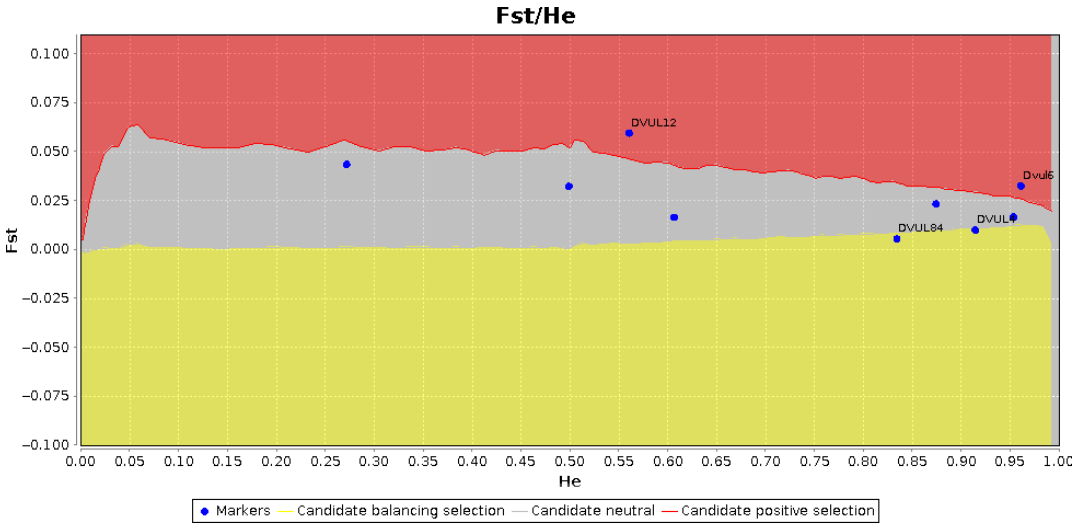

Fig. 2. Comparison of FST and H_e in polymorphic loci to identify outliers and potential candidates for selection using LOSITAN software). Graphical output shows the simulated confidence area for neutral loci (pale grey shading). Loci outliers are tagged with labels. Loci Dvul12 and Dvul6 are candidates for positive selection (red top area), whereas Dvul84 and Dvul4 loci are candidates for balancing selection (yellow bottom area).

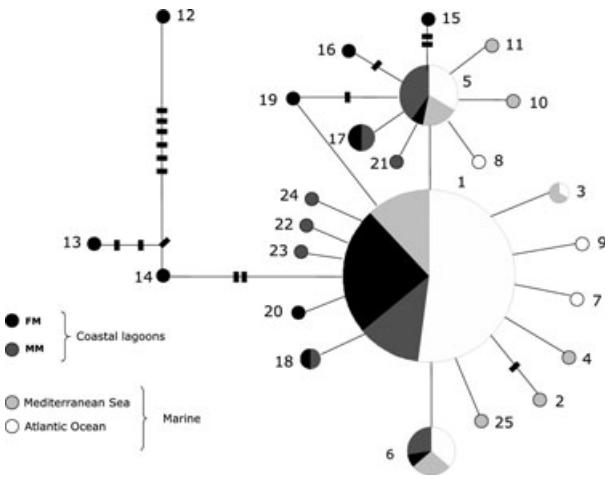

Fig. 3. Statistical parsimony network based on the cyt *b* sequences of *Diplodus sargus*. Each haplotype is defined by its corresponding number. The area of each circle is proportional to the number of individuals. Black bars represent putative mutational steps between haplotypes (black circles: Ria Formosa; grey circles: Mar Menor; light grey circles: Mediterranean Sea; white circles: Atlantic Ocean).

haplotype and Cytb-12 and Cytb-13 the most recent haplotypes. We adopted a conservative approach in the study of historic pattern from the network; this means that the haplotypes from some locations originated from more ancient sequences present in the same populations (*i.e.* Cytb-14 and Cytb-20 haplotypes from Ria Formosa arose from the Cytb-1 haplotype in Ria Formosa and we therefore rejected another geographical origin of these haplotypes).

Discussion

High genetic variation and low to moderate nucleotide diversity were found in all samples analysed for cyt *b* mtDNA marker and the microsatellite loci also showed high levels of polymorphism in *Diplodus sargus,* which is consistent with the previous data on this species (González-Wangüemert *et al.* 2010, 2011). The microsatellite analysis has showed deviations from Hardy–Weinberg (H–W) equilibrium in several loci that could be attributed to the presence of null alleles in some loci (Dvul61, Dvul6 and Dvul4). However, H–W deviations were found again when we eliminated these three loci of the analysis (data not showed). Other reasons for this disequilibrium include inbreeding, the Wahlund effect, selection against heterozygotes or strong directional selection as a consequence of the geographical isolation of some populations (Zouros & Foltz 1984; Mamuris *et al.* 1998; Rossi *et al.* 1998; González-Wangüemert *et al.* 2004a). Inbreeding is not likely considering the characteristics of *D. sargus* life strategy (spawning occurs in the open sea and they have pelagic eggs and larvae). Walhund effects due to the recruitment of genetically variable cohorts of larvae (González-Wangüemert *et al.* 2007) may represent an alternative, but not necessarily exclusive, hypothesis for heterozygote deficiency in populations of *D. sargus.* The role of selection can not be excluded based on our analysis, which detected positive selection on cyt *b* gene and two microsatellite loci. Selection could add to genetic drift because 'diverse groups' may respond in different ways to the extreme environmental conditions. There are

indeed an increasing number of studies showing that many polymorphisms and genetic clines are maintained by selection driven by environmental factors, primarily salinity and temperature in marine and brackish habitats (Eanes 2002; Gysels *et al.* 2004a; Cimmaruta *et al.* 2005; González-Wangüemert *et al.* 2006, 2009; Vergara-Chen *et al.* 2010a,b; Angeletti *et al.* 2010; Giménez-Casalduero *et al.* 2011).

It is important to stress that the highest values of genetic diversity measured as number of haplotypes, exclusive haplotypes and singletons, haplotype and nucleotide diversities, number of alleles and exclusive alleles, and heterozygosity, were recorded in both coastal lagoons (Mar Menor and Ria Formosa) for both mitochondrial and nuclear markers. Several authors have described that the species inhabiting temporally variable or spatially heterogeneous environments exhibit higher levels of genetic variability than those from less variable environments, which allows them to adapt better (Rand *et al.* 2002; Veliz *et al.* 2004; Garant *et al.* 2007; Iannotta *et al.* 2009; Richards *et al.* 2010). In fact, this higher genetic diversity in coastal lagoon populations has already recorded for some species using different molecular markers (Stefanni & Thorley 2003; González-Wangüemert *et al.* 2004b, 2006a; Gysels *et al.* 2004b; Vergara-Chen *et al.* 2010a,b).

We have detected deviations from the neutral model in some localities using Tajima's D and Fu's F_S tests. However, it is very difficult to distinguish between the contributions of natural selection and demographic history because variation patterns of neutral DNA sequence that are closely associated with a site that has undergone a recent adaptive substitution or 'selective sweep', are similar to those in an expanding population. It is important to stress that only the Ria Formosa sampling site, the most environmentally stressful site, showed significant values for both tests. Also, the AMOVA detected differences between groups (coastal lagoon and marine) close to significance. In fact, only two haplotypes were detected in both coastal lagoon sampling sites, and these locations (Mar Menor and Ria Formosa) showed the highest number of singletons, some of them with a high number of mutations, as has already been described for other Mar Menor populations (*Pomatochistus marmoratus*, control region, Vergara-Chen *et al.* 2010a; *Holothuria polii,* 16S gene, Vergara-Chen *et al.* 2010b).

The mitochondrial genome of animals is maternally inherited, generally non-recombining with other mitochondrial lineages, and contains 13 protein-coding genes very important to the fitness of individuals (Boore 1999). The mitochondrial genome is generally thought to evolve primarily under constant purifying selection, although the possibility of directional selection is gaining wider acceptance (Gerber *et al.* 2001; Bazin *et al.* 2006; Meiklejohn *et al.* 2007; Oliveira *et al.* 2008). Our results could support the hypothesis of selection (positive and balancing) as the driver of the genetic differences between coastal lagoon and marine samples, as demonstrated in several studies (Guinand *et al.* 2008; Blel *et al.* 2010). If selection is strong enough, differentiation may occur over relatively fine spatial scales, even in the face of considerable dispersal and gene flow (Conover *et al.* 2006; Sanford & Kelly 2010). In this case, differentiation is maintained by strong selection following dispersal (*i.e.* by purifying selection acting in each generation) (Schmidt & Rand 2001; Sanford & Kelly 2010).

Purifying selection is likely to be particularly important at very local spatial scales that are well within the dispersal range of the study organism (Schmidt *et al.* 2000; Hays 2007; Sherman & Ayre 2008). Although purifying selection may explain the observed genetic differentiation between populations from the lagoon, such a system could be best characterized as a balanced polymorphism rather than a strict local adaptation in which the genetic variation is maintained by balancing selection so that no single allele or haplotype always has the highest fitness (Hays 2007; Somero 2010). However, it is clear that genetic divergence can arise via purifying selection (Conover *et al.* 2006; Marshall *et al.* 2010) and, as result, adaptive differentiation among populations is expected to occur across a broad range of spatial scales and life histories (Sanford & Kelly 2011). Selection acting on early life stages may be strong enough to overcome even high rates of migration (Hellberg *et al.* 2002). Therefore, the genetic structure of a population under selective pressure will depend, among other factors, on the spatio-temporal distribution of the disturbance factors exerting the selection.

Considering the life history of white seabream, selection may act on early life stages (coastal lagoon recruits) in each generation. White seabreams behave as 'cyclic migrants', migrating into lagoons after metamorphosis and spending the early stages of their life cycle in these environments. Every year the sparid fauna migrate back to sea; the forces regulating this outward migration can either be intrinsic (reproduction) or linked to variations of abiotic factors (*i.e.* fluctuating temperature and salinity) (Ardizzone *et al.* 1988). A recent study carried out in Fogliano and Caprolace coastal lagoons (Mariani 2006) determined that the white seabream (*D. sargus*) stayed in the lagoon on average 1 year longer than the other sparids, whose populations encompassed mostly young fish from that year. This finding provided an interesting view of the adaptive potential of *D. sargus*. Even though a small proportion of the population left the lagoon after 6 months, possibly owing to temperature decrease, the majority of *D. sargus* seem capable of withstanding winter temperatures. They then benefit from the lagoon trophic substratum for a second warm

season and leave the lagoons in their second year with a size of 17–18 cm, which is larger than the average size of white seabream aged 1 year. The average size of *Diplodus sargus* at maturity is 20–25 cm and this is normally not attained before the third year (Martínez-Pastor & Villegas-Cuadros 1996), whereas the specimens are theoretically ready to spawn in spring of their second year, although they are not capable of breeding inside lagoons. The spawning time of this species is known to be extremely extended, from March to June (Bauchot & Hureau 1986), and growth rates are very variable (Gordoa & Molí 1997; González-Wangüemert *et al.* 2006b; Mariani 2006). *Diplodus sargus* may remain in coastal lagoons, thereby decreasing its mortality rate and improving the heritability of its genes. Thus the increased time in Mar Menor and Ria Formosa coastal lagoons with different temperatures and salinities could allow fitness selection and the maintenance of the exclusive haplotypes and genotypes favouring the 'divergence-with-gene flow'.

Conclusions

The highest values of genetic diversity of the white seabream were recorded in both coastal lagoons (Mar Menor and Ria Formosa) for mitochondrial and nuclear markers, which would favour a high potential for adaptation in populations inhabiting these stressed habitats. Some genetic features from coastal lagoon populations support the existence of selection. Future research on a higher number of species with different life strategies and the utilization of additional molecular ecology tools will be necessary to corroborate these results and to understand the evolutionary role of coastal lagoons.

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