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Genetic diversity of *Pomatoschistus microps* (Perciformes: Gobiidae) in ecologically differentiated estuarine systems

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Abstract. A landscape genetics approach was applied to common goby (*Pomatoschistus microps*) sampled from three estuaries (six sites) of the Portuguese coast. Individuals of each site were genotyped for eight microsatellite loci and levels of genetic diversity and differentiation were correlated to present-day estuarine characteristics and historical events. A general ecological state for each sampling site was obtained from a principal component analysis (PCA) applied to estuarine geomorphologic characteristics and levels of heavy metals and total polycyclic aromatic hydrocarbons contamination. Genetic diversity was higher than that previously reported for common goby in the Atlantic and Mediterranean. F_{ST} were generally very low (0.000-0.049), as well as Nei's genetic distances (0.000-0.167), although the later were statistically significant. Estuarine geomorphology and heavy metal contamination contributed the most to estuarine ecological differentiation but no trend was detected in the relationship between these characteristics and samples' genetic diversity. Mantel tests also revealed no significant relationships between geographic, genetic and ecological distances. Null alleles only contributed to explain significant Hardy-Weinberg departures in two of the eight loci scored, although disequilibria were detected in at least two loci per sample. Notwithstanding its exploratory character, results suggest an important role for historical factors in the timing and direction of *P. microps* colonization of the Portuguese estuaries. Environmental variation and *P. microps* ability to cope with it are also structuring factors in establishing and maintaining the patchy genetic diversity detected in the studied estuaries.

Key words: common goby, contamination, estuarine geomorphology, historical factors, microsatellites, landscape genetics

Introduction

The role of historical events in shaping genetic variability, specially last glacial maximum (LGM), has been relatively well studied (e.g. Hewitt 2000, 2004 and references therein), whereas that of extant local landscape and environmental factors has only recently attracted some attention (e.g. Costello et al. 2003, McCairns & Bernatchez 2008, Haponski et al. 2009, Pease et al. 2009). By combining molecular tools used in population genetics with the analyses of ecological biogeography and landscape ecology, Manel et al. (2003) developed a methodology called landscape genetics that correlates population genetic differences with concomitant discontinuities in environmental and/or landscape features. Indeed, it allows the detection of the relative influence of

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historic and contemporary processes in the observed genetic patterns, including identifying barriers to gene flow and the temporal and spatial scales of gene flow in relation to landscape features (McCairns & Bernatchez 2008, Haponski et al. 2009).

Concordant patterns of genetic and environmental differentiation, independent of geographic distance and historical factors have been identified in coastal and estuarine environments (e.g. McCairns & Bernatchez 2008, Haponski et al. 2009, Landínez-García et al. 2009, Olson et al. 2009), suggesting that environmental heterogeneity within these systems might be sufficient to lead to local adaptations.

Estuarine systems, in particular, have been subjected to significant anthropogenic pressures, a factor recognized to have an impact on the genetic structure of natural populations that should, therefore, be monitored (Pampoulie et al. 2004, Chung et al. 2008). Portuguese estuaries are subjected to distinct anthropogenic and natural pressures (Vasconcelos et al. 2007) and the available diversity of habitats within and between them supports different fish assemblages (França et al. 2009). In addition to differences in area, river flow, volume, mean depth, residence time and tidal range, the three largest estuarine systems along the western Portuguese coast (Ria de Aveiro, Tejo and Sado) are also characterized by the distinct anthropogenic activities developed within each transitional system: whereas fishing and aquaculture are very intense in Ria de Aveiro and Sado, Tejo is mostly affected by human population and industrial activities, both associated to organic and heavy metal pollution. Sado is also affected by agriculture activities mainly rice production, which uses high quantities of pesticides and fertilizers (Vasconcelos et al. 2007). All these characteristics contribute to the different availability and quality of saltmarsh, intertidal and subtidal habitats. This is reflected on the richness and diversity indices of fish assemblages measured in the three estuaries, with Tejo presenting the lowest values of these indices and Ria de Aveiro usually presenting the highest (França et al. 2009).

On the other hand, the geomorphologic features of each estuary might also influence their fish assemblages. Tejo and Sado estuaries originated about 18000 years ago, after the LGM (Dias et al. 2000). Most extant estuarine populations are, in fact, thought to have their origin in populations that took refuge in non-glaciated areas and latter expanded to these newly formed systems (Olson et al. 2009). Following a deglaciation period (16000 to 13000 years ago) and the re-emergence of the Gulf Stream (13000 to 11000 years ago) the rapid rise of sea level, from about –140 m (close to the shelf break) to its present day level (about 6000 years ago) (Dias et al. 2000), facilitated the sedimentation in Tejo and Sado estuaries. Contrasting with these older estuaries is Ria de Aveiro. Its origin dates back to the period between the 15th and 18th centuries, due to the Little Ice Age climatic events and to the deforestation and increased agricultural land use, both favouring sedimentation in Aveiro's coastal area (Dias et al. 2000).

In addition to the age, configuration and environmental differences observed between Ria de Aveiro, Tejo and Sado estuaries, three submarine canyons cutting the Portuguese shelf between these estuaries might also contribute to their distinct fish assemblages by limiting exchanges and migrations, including gene flow, between the three systems. Studies on population differentiation of estuarine and coastal fishes sampled in these estuaries have, indeed, suggested the canyons to influence their genetic structure, since low but significant values of genetic differentiation have been found in several fish species with different life cycles and ecological demands (Cabral et al. 2003, Pinheiro et al. 2005, Marques et al. 2006).

In order to assess the influence of estuarine characteristics on population structure and on their genetic exchanges, samples of common goby *Pomatoschistus microps* (Krøyer, 1838) from Ria de Aveiro, Tejo and Sado were subjected to microsatellite analyses. *P. microps* is a common fish species in Portuguese estuaries (França et al. 2009). Individuals are thought to be extremely sedentary (Berrebi et al. 2009), burrowing in rock crevices or under shells. This is particularly increased in males during spawning season: the female attaches the eggs to the ceiling of the nest built by the male, which ventilates and guards the eggs until hatching (Kvarnemo et al. 1998 and references therein). Because females spawn several times during the reproduction season, the period of paternal care is long and might involve guarding several clutches, contributing to reduce goby movements for feeding and leading to the rather frequent partial clutch cannibalism observed in common gobies (Kvarnemo et al. 1998). Although Atlantic individuals might migrate towards the sea for reproduction (Bouchereau & Guelorget 1997), Mediterranean individuals seem to spend all their life cycle, including reproduction, in estuaries or in coastal lagoons (Pampoulie et al. 2000).

Recent results support a pattern of multiple colonization events of Atlantic estuaries and Mediterranean lagoons after the LGM (Gysels et al. 2004, Berrebi et al.

2009). Pleistocene glaciations, by lowering sea level and drying out lagoons, limited estuarine connectivity which had two contrasting effects: the increase of genetic structure and the decrease of genetic diversity (Durand et al. 2005, Olson et al. 2009). As so, if Pleistocene glaciations are the major factor shaping the present genetic identity of *P. microps* in Ria de Aveiro, Tejo and Sado estuaries low genetic structure is expected. In contrast, if contemporary estuarine characteristics are more important in determining gene flow, population's isolation is expected and its effect should be reflected in the correlation between geographic and genetic distances.

In the present survey, analyses of the levels of genetic diversity and differentiation at eight microsatellite loci were conducted for six *P. microps* samples to verify: 1) if contemporary estuarine characteristics, such as the proportion of saltmarsh and intertidal areas and the levels of heavy metal contamination, are correlated with genetic variability and differentiation; and 2) if the geological history of estuaries and their isolation have promoted genetic differentiation.

Material and Methods

Study area and sampling

This study was conducted on three estuaries located on the western coast of Portugal (Fig. 1) with different geomorphologic and hydrologic characteristics (Table 1): Ria de Aveiro is a shallow coastal lagoon permanently connected to the sea and presenting a complex array of channels, whereas Tejo and Sado are the two largest estuaries in Portugal comprising wide estuarine bays; still, Tejo is not directly subjected to the oceanic waves' regime due to a narrow bedrock tidal inlet (Vis et al. 2008) and Sado is also protected from wave action by the Tróia peninsula. Located around Lisboa (the largest city in Portugal), Tejo is exposed to higher urban and industrial pressures (Vasconcelos et al. 2007) when compared to the other two estuaries, resulting in the generally higher heavy metal concentration observed in this estuary (Table 1). The aquaculture facilities that occupy 313 ha in Ria de Aveiro and 519 ha in Sado estuary also contribute to differences in anthropogenic pressures between the three estuaries (Vasconcelos et al. 2007).

Two sampling sites within each estuary were selected in order to integrate the effects of environmental heterogeneity on local adaptations (Fig. 1, Table 1). *P. microps* were collected in the six sampling sites during July 2009 using a beam trawl or a beach seine, depending on local features and habitat sampled. For the preliminary analyses presented here, 90 individuals were selected. Although *P. microps* is a common species in Portuguese estuaries (França et al. 2009), its abundance was not equally high in the three habitats (saltmarsh, intertidal, subtidal) sampled during the same tide, and a compromise had to be made between the ideal number of individuals to use in such a study and their identical time and place of collection. As so, only 15 individuals per estuarine site were screened for genetic diversity. Fin clips of each of individual were immediately excised after capture and preserved in absolute ethanol for DNA extraction.

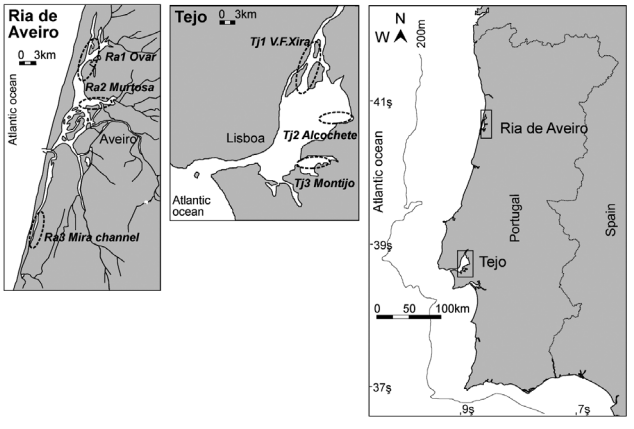

Fig. 1. *Sampling sites of* Pomatoschistus microps *within the three estuaries (Ria de Aveiro, Tejo and Sado), along the Portuguese coast. Acronyms for sampling sites are: Ria de Aveiro (Ovr – Ovar; Mir – Mira Channel); Tejo estuary (Vfx – Vila Franca de Xira; Alc – Alcochete); Sado estuary (Gam – Gâmbia; Car – Carrasqueira).*

Molecular methods

Eight pairs of microsatellite primers were tested: *Pmic2*, *Pmic3*, *Pmic7*, *Pmar3*, *Pmar5*, *Pmar8* (Berrebi et al. 2006), *Pmin5* (Jones et al. 2001) and *Pmin6* (Pampoulie et al. 2004).

Microsatellite PCR reactions (10 µL final volume) were carried out in an Eppendorf Mastercycler® according to the conditions proposed by Berrebi et al. (2006). Volumes of 2 µL of PCR product from each individual were then loaded onto an 8 % denaturing polyacrylamide gel (Bio-Rad®) and visualized with a FMBIO® fluorescent imaging system (HITACHI). Allele sizes were determined using a fluorescently labelled ladder of known size (Promega™) with the FMBIO® Analysis 8.0 image analyser software. The maximum frequencies of microsatellites scoring errors due to stuttering artefacts, large alleles' dropout and null alleles were estimated using Micro-Checker 2.2.3 (van Oosterhout et al. 2004).

Table 1. *Main geomorphologic and hydrologic characteristics of the Portuguese estuaries and sampling sites considered in the present study and their levels of sediment chemical contamination. TA – total area, RF – river flow, RT – residence time, CV – catchment volume, TR – tidal range, Salt – saltmarsh area, Inter – intertidal area, SubS – subtidal soft substrate area, SubH – subtidal hard substrate area, O – dissolved oxygen, T – temperature, C – conductivity, S – salinity, D – depth, Cd – cadmium, Cu – copper, Zn – zinc, Ni – nickel, Pb – lead, Cr – chromium, Hg – mercury, tPAH – total polycyclic aromatic hydrocarbons.*

Estuary	Site	TA[1,2] (km^2)	RF[1,2] ($m^3\ s^{-1}$)	RT[1,2] (days)	CV[1,2] ($10^6\ m^3$)	TR[1,2] (m)	Salt[2] (km^2)	Inter[2] (km^2)	SubS[2] (km^2)	SubH[2] (km^2)	O ($mg\ l^{-1}$)	T (°C)
Ria de Aveiro	Ovar	74	40	17	84	3.0	22	11	41	0	7.15	17.48
	Mira channel										8.66	18.49
Tejo	Vila Franca de Xira	320	300	25	1900	2.6	13	108	189	10	6.20	17.29
	Alcochete										5.13	16.62
Sado*	Gâmbia	180	40	30	500	2.7	11	76	76	0	5.53	17.80
	Carrasqueira										5.21	18.00

Table 1. *continued*

Estuary	Site	C ($ms\ cm^{-1}$)	S	D (m)	Cd[3] ($mg\ g^{-1}$)	Cu[3] ($mg\ l^{-1}$)	Zn[3] ($mg\ l^{-1}$)	Ni[3] ($mg\ l^{-1}$)	Pb[3] ($mg\ l^{-1}$)	Cr[3] ($mg\ l^{-1}$)	Hg[3] ($mg\ l^{-1}$)	tPAH[3] ($ng\ g^{-1}$)
Ria de Aveiro	Ovar	35.33	24.40	1.58	2.09	25.46	348.36	20.24	41.54	15.90	0.03	27.68
	Mira channel	25.25	15.82	1.76	1.58	20.02	151.00	15.69	33.82	13.30	0.02	10.99
Tejo	Vila Franca de Xira	24.44	15.01	3.40	1.44	27.83	178.59	26.32	74.32	21.91	0.11	56.31
	Alcochete	27.68	19.56	1.26	1.97	27.58	190.99	21.74	76.99	19.56	0.17	45.94
Sado*	Gâmbia	49.76	34.54	2.18	1.49	45.71	162.17	24.13	44.12	28.86	0.05	19.10
	Carrasqueira	48.93	34.55	2.49	1.19	39.69	135.49	28.25	43.97	30.30	0.03	26.38

*[1] – Vasconcelos et al. (2007), [2] – França et al. (2009), [3] – Fonseca et al. (2011); * sediment chemical contamination data in Sado estuary were obtained in the present study.*

Population structure analyses

The total number of alleles (A), the observed heterozygosity (H_o) and the unbiased expected heterozygosity (H_{nb}) (Nei 1978) were calculated for each sample together with allele frequencies. Inbreeding coefficient (F_{IS}, Wright 1969) was estimated using the *f* parameter of Weir & Cockerham (1984) to test the departures from Hardy-Weinberg (HW) equilibrium within each sample. Fixation index (F_{ST}, Wright 1969) representing the inter-sample differentiation was estimated using the θ parameter of Weir & Cockerham (1984). Nei's genetic distance (*D*, Nei 1978) was also calculated in order to quantify the genetic divergence between sample pairs. Significance of F_{IS}, F_{ST} and *D* values were obtained by random permutation procedures (5000 allele permutations within samples for F_{IS} and 5000 individual permutations between samples for F_{ST} and *D*). Linkage disequilibrium between each pair of loci or alleles was also assessed according to Black & Krafsur (1985) in order to check for the independency of the loci amplified. All calculations were performed using Genetix 4.05 (Belkhir et al. 2004).

Bottleneck 1.2.02 (Cornuet & Luikart 1997, Piry et al. 1999) was used to test if the six *P. microps* sampled populations have been subject to recent bottleneck events, as suggested by the HW disequilibria detected in some loci (see the results section). The significance of the test was assessed with the Wilcoxon test included in the software, which provides relatively high power and can be used with as few as four polymorphic loci and any number of individuals (Piry et al. 1999). Two models of evolution were used for microsatellites: infinite alleles model (IAM) and stepwise mutation model (SMM), considered as the two extreme models able to check all the hypotheses for this study.

Table 2. *Observed (H_o) and unbiased (H_{nb}) heterozygosity (Nei 1978), F_{IS} values and the total number of alleles (A) found for each microsatellite locus within each* Pomatoschistus microps *sample. Significance of Hardy-Weinberg equilibrium was determined with F_{IS} values (Weir & Cockerham 1984) (* p < 0.05, ** p < 0.01). The total number of different alleles in each locus (a) is also shown.*

Locus (a)	Ria de Aveiro								Tejo estuary							
	Ovar				Mira Channel				Vila Franca de Xira				Alcochete			
	H_o	H_{nb}	F_{IS}	A	H_o	H_{nb}	F_{IS}	A	H_o	H_{nb}	F_{IS}	A	H_o	H_{nb}	F_{IS}	A
Pmic2 (44)	0.69	0.91	0.25	13	1.00	0.99	–0.01**	25	0.64	0.90	0.30*	14	0.85	0.93	0.10	14
Pmic3 (12)	0.55	0.83	0.35	6	0.55	0.82	0.35	7	0.38	0.84	0.55**	6	0.50	0.83	0.40	7
Pmic7 (3)	0.46	0.49	0.06	2	0.25	0.49	0.50	2	0.77	0.49	–0.60**	2	0.71	0.55	–0.32	3
Pmar3 (28)	0.60	0.93	0.36*	15	0.80	0.92	0.14	15	1.00	0.94	–0.06**	15	0.57	0.94	0.40**	13
Pmar5 (21)	0.50	0.67	0.26	5	0.69	0.86	0.21	12	0.73	0.71	–0.03	6	0.71	0.79	0.10	8
Pmar8 (2)	0.25	0.23	–0.10**	2	0.15	0.15	–0.04**	2	0.40	0.34	–0.20**	2	0.21	0.20	–0.08**	2
Pmin5 (22)	0.86	0.89	0.04	12	0.87	0.79	–0.11	8	0.93	0.90	–0.03	13	0.87	0.85	–0.02	11
Pmin6 (23)	0.20	0.63	0.69**	7	0.33	0.76	0.57**	9	0.42	0.78	0.48**	10	0.53	0.71	0.25	9
Average	0.51	0.70	0.27**	7.75	0.58	0.73	0.20**	10	0.66	0.74	0.11	8.50	0.62	0.72	0.15	8.37

Table 2. *continued*

Locus (a)	Sado estuary							
	Gâmbia				Carrasqueira			
	H_o	H_{nb}	F_{IS}	A	H_{oO}	H_{nb}	F_{IS}	A
Pmic2 (44)	1.00	0.96	–0.04**	18	0.79	0.93	0.16	16
Pmic3 (12)	0.38	0.83	0.55**	7	0.42	0.64	0.36	6
Pmic7 (3)	0.46	0.56	0.18	3	0.70	0.48	–0.50**	2
Pmar3 (28)	0.86	0.94	0.10	15	0.79	0.95	0.18	16
Pmar5 (21)	0.69	0.76	0.10	9	0.69	0.88	0.22	12
Pmar8 (2)	0.38	0.32	–0.20**	2	0.08	0.08	–0.00**	2
Pmin5 (22)	0.79	0.77	–0.03	10	0.85	0.90	0.06	11
Pmin6 (23)	0.31	0.82	0.63**	7	0.33	0.89	0.64**	11
Average	0.61	0.75	0.19**	8.87	0.58	0.72	0.20**	9.50

The Bayesian model-based clustering method of Pritchard et al. (2000), as implemented in the program STRUCTURE 2.1, was employed to infer population structure from the microsatellite data, without using prior information on the geographic origins of the samples. A pattern of admixture was evaluated by varying the number of groups (K) in the dataset and assigning proportions of each individual to these groupings, assuming HW equilibrium within clusters (i.e., samples), and linkage equilibrium between loci within samples. A likelihood function of the genotypic data of the sampled individuals (*90*) was estimated for different values of K ($2 \leq K \leq 10$) to infer the likely number of populations in the data and to approximate the posterior probability distribution of K, given the data. To guarantee convergence to a stationary distribution three separate runs, each with a burn-in of 50000 followed by 100000 iterations, were performed for each value of K. The isolation-by-distance (IBD) hypothesis was tested using $F_{ST}/(1\text{-}F_{ST})$ as the genetic distance (Rousset 1997) and the logarithmic transformation of geographic distances. These corresponded to the shortest straight distance between sites within estuaries and between sites in different estuaries. The test was conducted in GENETIX 4.05, using the Mantel test (Mantel 1967). Significance of the correlation between distances was evaluated by a permutation test, based on 5000 randomizations.

Genetic differentiation in relation to environmental conditions

Information on several environmental variables characterizing estuaries and sites within estuaries was gathered in order to determine the extent to which they correlate to the patterns of microsatellite variation. Data on estuarine total area, river flow, residence time (average time that a water molecule spends in the estuary), volume, tidal range and percentage of each habitat type (Table 1) were obtained from previous studies (Vasconcelos et al. 2007, França et al. 2009). Depth (m), water temperature (°C), salinity, conductivity (ms cm^{-1}) and dissolved oxygen (mg l^{-1}), taken in four sampling periods (January, April, July and October 2009), were averaged in order to obtain a value that better characterized each site. Average values of heavy metal and total polycyclic aromatic hydrocarbons (tPAHs*)* contamination in each site were used as environmental variables (Table 1). Values for Ria de Aveiro and Tejo estuaries were obtained from Fonseca et al. (2011) whereas those for Sado are first presented here, measured according to the methodology described in Fonseca et al. (2011).

The proportion of environmental variance explained by the environmental variables was evaluated by a principal components analysis (PCA) considering the eigenvectors of PCA on the correlation matrices of all environmental variables (Table 1). Similar to the method used by McCairns & Bernatchez (2008) and Pease et al. (2009), a general ecological state of each sampling site was defined based on their scores from the first and second eigenvectors (PC1 and PC2) of the PCA. These scores were then used to calculate the ecological distance between sites in Primer 5 (Clarke & Gorley 2001), based on a matrix of Euclidean distances. A Mantel test comparing these ecological distances vs. $F_{ST}/(1-F_{ST})$ (used as the genetic distance) was carried out in Genetix 4.05.

Results

Characteristics of microsatellite loci and population structure

According to the output of Micro-Checker, large alleles' dropout and stuttering artefacts were not detected in the microsatellite data set. Null alleles were only observed in *Pmar3* and *Pmin6*. The number of alleles per sample (A) ranged from 2 to 25 for the eight loci (Table 2), with the highest average value registered in Mira Channel (Ria de Aveiro). The total number of detected alleles in each locus (a) varied in a wider range – from 2 in *Pmar8* to 44 in *Pmic2*. Observed heterozygosity (H_o) was very variable between locus and populations: the lowest value (0.08) was obtained for locus *Pmar8* in Carrasqueira (Sado estuary) and the highest (1.00) for loci *Pmic2* in Mira Channel and Gâmbia (Sado estuary) and *Pmar3* in Vila Franca de Xira (Tejo estuary). Expected heterozygosity (H_{nb}) varied within the same range – the lowest value of this statistics (0.08) was also registered for *Pmar8* in Carrasqueira and the highest (0.99) for *Pmic2* in Mira Channel. With the exception of *Pmar5* and *Pmin5*, all loci presented significant heterozygote deficiency or excess in at least one population. *Pmar8* was the only locus where significant heterozygote excess was detected in all populations, which also shared the only two alleles detected for this locus (Table 2).

Nei's genetic distances (*D*) were very low but all statistically significant, except those between

Table 3. *Nei's genetic distance (Nei 1978) (below the diagonal) and F_{ST} values (above the diagonal) between pairs of* Pomatoschistus microps *samples.*

	Site	Ria de Aveiro		Tejo estuary		Sado estuary	
		Ovar	Mira Channel	Vila Franca de Xira	Alcochete	Gâmbia	Carrasqueira
Ria de Aveiro	Ovar		–0.001	0.000	–0.014	–0.001	0.018
	Mira Channel	0.021*		0.003	–0.007	–0.004	0.016
Tejo estuary	Vila Franca de Xira	0.017*	0.025*		0.002	0.009	0.049*
	Alcochete	0.000	0.000	0.019*		–0.005	0.014
Sado estuary	Gâmbia	0.017*	0.009*	0.044*	0.002*		0.008
	Carrasqueira	0.070*	0.064*	0.167*	0.054*	0.045*	

** indicates significant values ($p < 0.05$).*

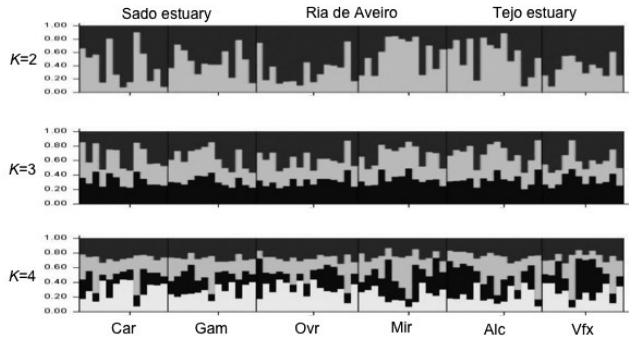

Fig. 2. *Estimated structure. Each individual is represented by a thin vertical line, which is partitioned into K segments of different colours that represent the individual's estimated membership fractions in K clusters. Vertical black lines separate individuals of different samples. Samples are labelled below the figure, with their estuarine affiliations above it: Ovr – Ovar; Mir – Mira Channel; Vfx – Vila Franca de Xira; Alc – Alcochete; Gam – Gâmbia; Car – Carrasqueira.*

Alcochete (Tejo estuary) and the two populations from Ria de Aveiro, which presented the lowest values (= 0.000, Table 3). The highest *D* was obtained between Vila Franca de Xira and Carrasqueira (0.167). No linkage genotypic disequilibrium was found between loci overall samples or between loci per sample pairs after Bonferroni corrections for multiple tests ($p > 0.05$ in all 196 tests). F_{ST} values were also very low (Table 3) ranging from 0.000 (between Ovar and Vila Franca de Xira) to 0.049 (between Vila Franca de Xira and Carrasqueira), which was the only significant value.

No indication of recent reductions in population size (bottleneck) was found in any sample, considering all microsatellites. Tests for heterozygosity excess produced no significant *P*-values, although Vila Franca de Xira (Tejo estuary) and Carrasqueira (Sado estuary) presented values close to significance ($p = 0.95$) under IAM. This is consistent with the similar levels of variability observed in all samples ($0.51 < H_o < 0.66$).

No clusters were evidenced by STRUCTURE analyses of the eight microsatellites (Fig. 2, showing *K* between 2 and 4). All individuals presented a similar probability of belonging to any of the *K* groups tested (i.e. from 2 to 10), which is characteristic of a global homogeneous sample.

The Mantel test applied to genetic vs. geographic distances revealed no significant relationships between the two matrices (Z = 0.371), therefore rejecting the hypothesis of IBD.

Genetic differentiation and environmental factors

No significant trend was detected in the variation of heterozygosity, inbreeding coefficient and the total number of alleles according to estuarine geomorphology, hydrologic pattern or level of sediment contamination, despite the lowest average values of H_o, H_{nb} and A (0.51, 0.70 and 7.75, respectively) registered in the smallest estuary, i.e. Ria de Aveiro (Tables 1 and 2). The lowest average values of genetic statistics were generally found in Ovar, with the exception of F_{IS} that was recorded in Vila Franca de Xira, whereas the lowest values of sediment contamination were observed in Mira Channel. Similarly, and although the highest values of H_o were found in Vila Franca de Xira (Table 2), which is one of the sampling sites within the largest estuary, i.e. Tejo estuary (Table 1), for some of the loci analyzed H_o was higher in Mira Channel located in Ria de Aveiro, the smallest estuary analysed (Table 2). Average values of H_{nb} and A were higher in Gâmbia and Carrasqueira in the Sado system (0.75

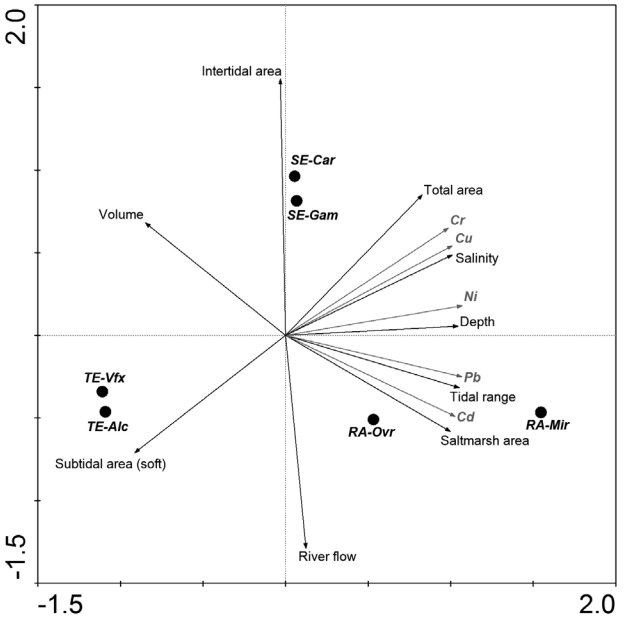

Fig. 3. *Biplot of* Pomatoschistus microps *samples (filled circles) and the environmental variables (arrows) contributing the most to their differentiation along the first (PC1, horizontal axis) and second (PC2, vertical axis) principal components. Grey arrows represent heavy metals (Cd – cadmium, Cr – chromium, Cu – copper, Ni – nickel, Pb – lead). Positions of samples are based on their respective PC1 and PC2 scores. Acronyms for sampling sites are: RA – Ria de Aveiro (Ovr – Ovar; Mir – Mira Channel); TE – Tejo estuary (Vfx – Vila Franca de Xira; Alc – Alcochete); SE – Sado estuary (Gam – Gâmbia; Car – Carrasqueira).*

and 9.50, respectively), where conductivity, salinity and contamination by copper, nickel and chromium were also higher (Table 1). Overall F_{IS} values were higher in Ovar, where the lowest values of all other genetic statistics were found.

PCA results revealed that 88.5 % of the cumulative variation in environmental data was explained by the first two components (72.5 % PC1 and 16.0 % PC2) (Fig. 3). PC1 was mostly related to estuarine geomorphology (total area, volume and depth of the estuary) and levels of heavy metal contamination while PC2 was mainly related to the availability of saltmarsh, subtidal and intertidal habitats and river flow. Sites within each estuary were grouped in the diagram and each estuary was mainly differentiated from the others by the availability of a particular habitat: intertidal in Sado estuary, saltmarsh in Ria de Aveiro, subtidal in Tejo estuary. Whereas the larger catchment volume of Tejo estuary was important in its separation from the other two areas, Sado estuary's differentiation was related to the higher concentrations of copper, nickel and chromium found in this system (Table 1, Fig. 3). The larger tidal range of Ria de Aveiro and the higher concentration of cadmium found within this estuary were also important for its environmental distinction from the other two areas.

The Mantel test applied to genetic vs. ecological distances revealed no significant relationships between the two matrices (Z = 0.357), therefore rejecting the hypothesis of "isolation by ecological distance".

Discussion

Despite inhabiting environmental differentiated sites, *P. microps* samples presented similar levels of genetic variability ($0.51 < H_o < 0.66$), all much higher than those previously reported on allozymes for this species along the northeast Atlantic Ocean and Mediterranean Sea ($0.066 < H_o < 0.135$, Gysels et al. 2004). However, heterozygosity levels found in the present study were lower than that reported by DeWoody & Avise (2000) for populations of marine fish, using microsatellites (0.79). According to the results of Gysels et al. (2004), the sample from Faro (southern Portuguese coast) was the most diverse population, presenting the highest values of observed and unbiased expected heterozygosity and mean number of alleles (0.135, 0.139 and 1.769, respectively). Although average heterozygosity values depend on the number and type of loci analyzed, and comparisons to other studies must be cautious, especially when different categories of markers (allozymes or mitochondrial DNA vs. microsatellites) are employed, present results support the high levels of genetic diversity found by Gysels et al. (2004) in the Portuguese coast. Because Ria de Aveiro, is only a few hundred years old (Dias et al. 2000), the generally lower levels of genetic diversity found here, compared to those from Tejo and Sado, could correspond to a recently established population originating from individuals that migrated from another population. In fact, and although based in small sample sizes, levels of genetic diversity found in this study fit to the model of a northward gene flow and colonization from the south along the Portuguese coast, proposed by Gysels et al. (2004), with Tejo and Sado possibly acting as the source populations to Ria de Aveiro, the most northern estuary on this study. The low genetic distances (D) and F_{ST} values found between *P. microps* samples from the three estuaries, also suggest gene flow is maintained between them.

On the other hand, most values of D were significant and D and F_{ST} between Carrasqueira (Sado estuary) and Vila Franca de Xira (Tejo estuary) were much higher, and also significant, than those presented between other sample pairs, suggesting some level of population structuring. Although significant population differentiation has been reported elsewhere for *P. microps* at small geographic scales in the Atlantic and Mediterranean (Gysels et al. 2004, Berrebi et al. 2009), STRUCTURE analyses failed to reveal population sub-divisions, supporting that only one population unit is found on the studied area. While to be confirmed by larger sampling, the pattern of genetic differentiation of *P. microps* found here, patchy and not related to geographical distances, does not support, at least at this local scale, the model of isolation-by-distance referred by Gysels et al. (2004) for their north-eastern Atlantic samples. Moreover, this pattern of weak but significant genetic differentiation has been reported for other fish species inhabiting Sado and Tejo estuaries and the adjacent coastal areas, namely the sole species *Solea lascaris* (Risso, 1810), *Solea senegalensis* (Kaup, 1858) and *Solea solea* (Linnaeus, 1758) and the toadfish *Halobatrachus didactylus* (Bloch & Schneider, 1801) (Cabral et al. 2003, Pinheiro et al. 2005, Marques et al. 2006), thus suggesting Sado and Tejo could have played an important role in shaping the genetic diversity observed in these species, either due to the historical events or to the geomorphologic complexity of these estuaries.

From an historical perspective, a possible explanation for the higher genetic distances between Carrasqueira and Vila Franca de Xira, located in the innermost locations of Sado and Tejo estuaries, respectively, might be that these sites acted as refuges during

glaciations. Since the common goby only reproduces in shallow waters (Gysels et al. 2004), and is able to cope with salinity and temperature fluctuations (Dolbeth et al. 2007, Rigal et al. 2008), populations of *P. microps* could have been isolated in these areas during glaciations, and latter expanded to other estuarine locations and other estuaries. This is also in agreement with the recent results obtained for the phylogeography of the sand goby, *Pomatoschistus minutus* (Pallas, 1770), which indicated Tejo estuary as a southern refuge for this species during Pleistocene glaciations and a subsequent northward expansion (Larmuseau et al. 2009).

Significant heterozygote deficiency or excess was detected in all populations for at least one locus. Although null alleles can partly explain the heterozygote disequilibria observed in *Pmar3* and *Pmin6*, the tempo and mode of *P. microps* reproduction could be reflected in the inbreeding coefficients calculated, as the sedentary behaviour of this species might contribute to each sampling site matching a separate breeding unit and some parents might dominate the progeny – e.g. a single male might provide parental care for several broods of the same female, in space and time (Kvarnemo et al. 1998). On the other hand, the dispersal of larvae during their pelagic stage, and the several larvae supplies during the reproduction season might counteract the effect of inbreeding. As so, because the estuaries studied here are large, present high habitat diversity and different human and natural pressures (Vasconcelos et al. 2007, França et al. 2009) and *P. microps* is able to cope with different environmental perturbations (Pampoulie et al. 2000, Rigal et al. 2008), the HW disequilibria presented might be the earliest indication that populations could be adapting to the environmental characteristics of each site, also supported by the non-significant relation between genetic distances and ecological differences between sites.

The factors identified here as contributing the most to sampling site differentiation (e.g. habitat availability and sediment contamination) are amongst those referred to as the main environmental characteristics structuring *P. microps* populations (Dolbeth et al. 2007 and references therein). Several studies have investigated the relationship between exposure to contamination (by heavy metal or organic compounds) and shifts in genetic frequencies and variability (e.g. Heithaus & Laushman 1997, De Wolf et al. 2004, McMillan et al. 2006, Chung et al. 2008) based on the hypothesis that toxicants cause DNA damage producing variations in genotype frequencies and population bottlenecks. In the present study, none of the sampled sites consistently presented the highest levels of sediment contamination and no recent bottlenecks were detected amongst the six samples. On the other hand, the highest levels of heterozygosity were observed in Tejo estuary, which is characterized by a shallow and large bay with several islets and canals and vegetated and uncovered grounds, that provide a multiplicity of different habitats, when compared to Sado and Ria de Aveiro, suggesting that habitat diversity could be a major factor influencing genetic diversity in *P. microps*.

In fact, environments that require broader tolerance ranges, contribute to higher genetic variation in species able to cope with those fluctuations (Willis 1973), as is the case of the common goby (Pampoulie et al. 2000, Rigal et al. 2008). This ability to adapt to different environmental conditions, allied to a high genetic diversity and to the maintenance of gene flow between sampling sites within and between estuaries, could have resulted in the small genetic distances observed in this preliminary study. This low genetic differentiation between the three estuaries suggests deep canyons cutting the Portuguese shelf are not effective barriers to gene flow in *P. microps*, since their long-distance dispersal might be mostly effective during the pelagic larval stage (Gysels et al. 2004). Within estuaries, reproductive and thermal migrations, described for Atlantic common gobies by Bouchereau & Guelorget (1997), might be responsible for maintaining the connection between samples, as no true physical barriers exist between the sampled sites.

Hence, the analyses performed in this groundwork suggest that the patchy genetic diversity and low but significant genetic differentiation of *P. microps* along the Portuguese coast reported here result from a compromise between historical factors, extant environmental factors and biological constraints. Whereas the first, related to glaciations and post-glacial periods, might have established the colonization route of estuaries and the timing of this colonization, the environmental diversity within each estuary, and the ability of common goby to cope with it and disperse during its pelagic larval stage, contribute to maintain gene flow and a high genetic diversity. Still, differences in ecological conditions between sites might not be sufficient to cause genetic differentiation in individuals inhabiting these sites through directional selection and adaptation, or these might not be detected, in a scenario of constant gene flow. The patterns found should, therefore, be clearly

tested in future analyses with larger sample sizes, more sampling sites and using more genetic markers in order to provide further insight into the importance of environmental factors on genetic differentiation.

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