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Genetic and morphological differentiation of the Lusitanian toadfish (*Halobatrachus didactylus*) between estuarine and coastal areas in Portugal

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SUMMARY**:** The Lusitanian toadfish, *Halobatrachus didactylus* (Bloch and Schneider, 1801), is distributed from the Ghana coast to the Iberian Peninsula, being particularly abundant on the south coast of Portugal. The differentiation of this species along the Portuguese coast was assessed through the analysis of 10 samples, considering morphological characters (20 morphometric and 16 meristic) and genetic markers (10 allozymes, 11 *loci*). Southern samples included estuaries and their adjacent coastal areas, given that this species inhabits both environments, whereas western samples only comprised estuaries. Morphometric and meristic data discriminant analysis evidenced some differentiation between estuarine and coastal populations. This was not entirely corroborated by the genetic analysis, which showed an overall pattern of low F_{ST} (0.042) and Nei's genetic distance, even between geographically distant areas. However, higher values of these parameters were found between estuaries of the south coast and their adjacent coastal areas, suggesting that estuarine systems play a major role in such differentiation. Results are discussed regarding toadfish life-history pattern and Portuguese coast geomorphology, giving an insight into the biological and environmental factors influencing population sub-structuring.

Keywords: *Halobatrachus didactylus* differentiation, allozymes, morphology, population structure, coastal areas

RESUMEN: DIFERENCIACIÓN MORFOLÓGICA Y GENÉTICA DEL PEJESAPO (*HALOBATRACHUS DIDACTYLUS*) ENTRE ESTUARIOS Y ÁREAS COSTERAS DE PORTUGAL. **–** El pejesapo, *Halobatrachus didactylus* (Bloch y Schneider, 1801), está distribuido desde la costa de Ghana hasta la Península Ibérica, siendo particularmente abundante en la costa sur portuguesa. La diferenciación de esta especie a lo largo de la costa Portuguesa se ha evaluado a través del análisis de diez muestras, considerando caracteres morfológicos (20 características morfométricas y 16 merísticas) y genéticos (10 aloenzimas, 11 l*oci*). Hacia el sur, las muestras incluyen estuarios y sus áreas costeras adyacentes, ya que esta especie habita ambos ambientes, mientras que las muestras del oeste están relacionadas sólo a estuarios. El análisis discriminante de los datos morfométricos y merísticos evidenciaron diferenciación entre poblaciones de estuarios y costeras, lo cual no fue enteramente corroborado por el análisis genético, el cual mostró un patrón general de bajo F_{ST} (0.042) y distancia genética de Nei, incluyendo áreas geográficamente distantes. Sin embargo, valores más altos de estos parámetros fueron encontrados entre estuarios de la costa sur y sus áreas costeras adyacentes, sugiriendo que los sistemas de estuarios juegan un papel importante en tal diferenciación. Los resultados son discutidos considerando los patrones de historia de vida del pejesapo y la geomorfología de la costa portuguesa, dando una perspectiva de cómo los procesos biológicos y factores ambientales influyen la sub- estructuración poblacional.

Palabras clave: *Halobatrachus didactylus* diferenciación, alozimas, morfología, estructura poblacional, áreas costeras.

INTRODUCTION

The Lusitanian toadfish, *Halobatrachus didactylus* (Bloch and Schneider, 1801) (Batrachoididae) is a sedentary benthic species that lives down to about 50 m, inhabiting the eastern Atlantic from Ghana to the Iberian Peninsula (about 39ºN) (Roux, 1986). On the Portuguese coast this species is particularly abundant in estuaries south of Sado (38ºN) (Sobral and Gomes, 1997) and has a peculiar distribution:

on the western coast it occurs only in estuaries but on the south coast it occurs in both estuaries and coastal areas (Costa, 1993). Together with its distribution, this species' life history also suggests a low flux of individuals, even between close areas, since males nest under rocks or in crevices defending the clutch (Santos *et al.*, 2000). This is expected to result in a differentiation by genetic drift, inbreeding or, as suggested by Beheregaray and Sunnucks (2001) for species that inhabit coastal and estuarine regions, "divergence-with-gene-flow".

Like *H. didactylus*, the widely distributed sand goby, *Pomatoschistus minutus* (Pallas, 1770), inhabits both estuarine and coastal areas and adults have little or no migration. Research on the genetic diversity and differentiation of this species using allozymes suggested that the morphological differentiation observed in the lagoon of Venice was related to population structuring (Stefanni *et al.*, 1996; 2003). Studies conducted at four locations in the North Sea using microsatellite markers also revealed differentiation between estuarine, coastal and marine samples of *P. minutus* (Pampoulie *et al.*, 2004). Within the family Batrachoididae population sub-structuring has been described for *Opsanus tau* Linnaeus, 1758 and *Opasanus beta* Goode and Bean, 1879 inhabiting each side of cape Hatteras on the western Atlantic coast (Avise *et al.*, 1987). Considerable genotypic diversity and differentiation was observed within each species due to the constrained gene flux between populations.

In studies considering population differentiation the selection of marine areas where major biogeographic shifts occur are as important as species' life history and ecology (Nielsen *et al.*, 2004). The Portuguese coast is therefore a suitable area for the study of population interactions due to its transitional character as a border area between the warm-temperate and cool-temperate Atlantic zoogeographic areas. Here, Castilho and McAndrew (1998) found a significant population sub-structuring for *Dicentrarchus labrax* (Linnaeus, 1758) juveniles in five estuaries. However, for two species of soles with a similar life history pattern, *Solea solea* (Linnaeus, 1758) and *Solea senegalensis* Kaup, 1858, Cabral *et al.* (2003a) found a low genetic differentiation. These contradicting results suggest that, although estuaries may contribute to the genetic divergence observed in species occurring in estuarine and open-coast environments (Bilton *et al.*,

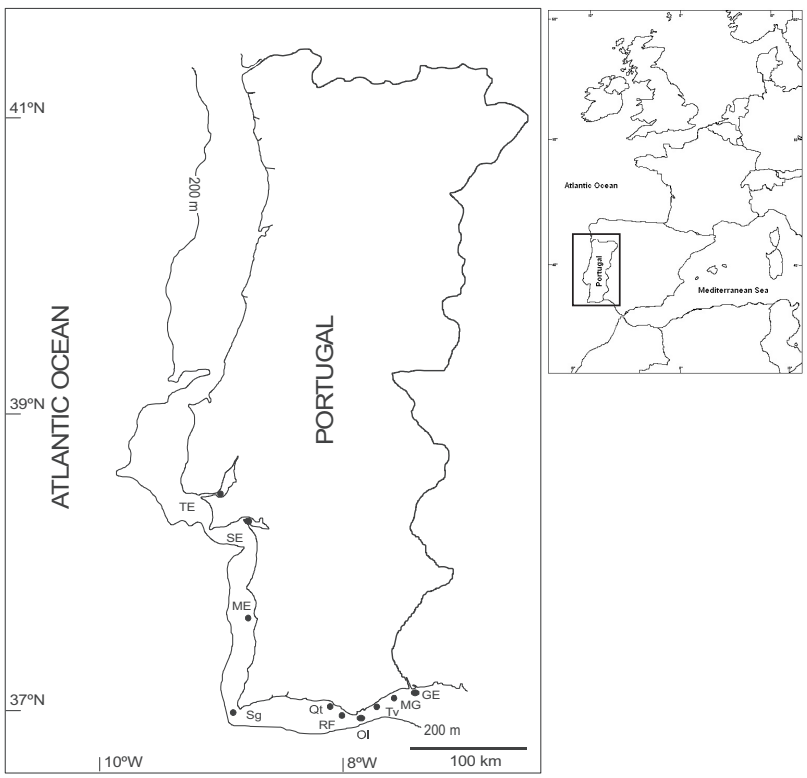

FIG. 1. – Location of *H. didactylus* sampling areas (*n* = sample size). TE, Tejo estuary (*n* = 52); SE, Sado estuary (*n* = 52); ME, Mira estuary (*n* = 35); Sg, Sagres (*n* = 36); Qt, Quarteira (*n* = 56); RF, Ria Formosa estuary (*n* = 46); Ol, Olhão (*n* = 52); Tv, Tavira (*n* = 53); MG, Monte Gordo (*n* = 35); GE, Guadiana estuary (*n* = 49).

2002), the differentiation patterns depend on whether the species only use them as a special habitat during their life cycle or they actually support distinct populations.

In the present study, morphological and enzymatic characters of *H. didactylus* inhabiting 10 areas along the Portuguese coast were used in order to: 1) identify and quantify the phenotypic and genotypic variability; 2) analyse the morphological and genetic differentiation of *H. didactylus*; and 3) infer the importance of estuaries in population structuring.

MATERIAL AND METHODS

Sampling procedures

A total of 466 individuals of *H. didactylus* were sampled from 10 areas along the Portuguese coast (Fig. 1). Sampling sites on the western coast (the Tejo, Sado and Mira estuaries) were widely separated, whereas most of those on the south coast comprised estuaries and their adjacent coastal areas. This set of samples allowed the detection of regional and local patterns of differentiation as well as the comparison between estuarine and marine environments. Beam trawl fishing was carried out during the non-matting period, between September and April 2000, and all individuals from each sample were caught in the same trawl. Liver and muscle tissue samples were taken from all specimens and stored at – 80ºC until electrophoretic analysis.

Morphological analysis

Characters easily repeatable from individual to individual, including distances between clearly recognisable landmarks of the species anatomy, were selected for the morphological analysis of the 10 samples. This resulted in a total of 20 morphometric—including horizontal as well as vertical dimensions of the body—and 16 meristic characters (Table 1). Analyses of these variables were carried out separately because they are different in their statistical nature and might therefore be responding differently to environmental and genetic factors (Ihssen *et al.*, 1981).

All morphometric characters, measured to the nearest 0.1 mm, were transformed to logarithms to approximate multivariate normality. Given that body measurements are generally strongly correlated with the individuals' total length (TL), morphometric characters were standardised to the overall mean total length using Hurlbut and Clay's (1998) expression:

$$M_C = M_X \cdot \left(\frac{\overline{TL}}{TL} \right)^b$$

in which TL is the total length, M_x is the original measurement, $\overline{TL}$ is the overall mean total length and b is the slope, within areas, of the geometric mean regression (Ricker, 1973) on the logarithms of M_x and TL. This regression model was chosen because any of the variables could be considered as depending on another. The resulting variables were then considered shape discriminators (Humphries *et al.*, 1981). After size effect removal, variables remaining highly correlated were considered redundant and eliminated from the analysis. Meristic

TABLE 1. – Morphometric and meristic variables considered in the morphological analysis of *H. didactylus*.

Morphometric	
Total length	TL
Standard length	SL
Length from the snout to the beginning of first dorsal fin	1DL
Length from the snout to the beginning of second dorsal fin	2DL
First dorsal fin length	D1
Second dorsal fin length	D2
Caudal fin length	CL
Length from the snout to the beginning of left pectoral fin	PPL
Length from the snout to the beginning of left ventral fin	PVL
Length from the snout to the beginning of the anal fin	PAL
Length of the left pectoral fin	LPL
Length of the left ventral fin	LVL
Anal fin length	AL
Distance between the first pair of nostrils	DN1
Distance between the second pair of nostrils	DN2
Inter-orbital distance	IOD
Left eye diameter	LED
Head width (measured between the most distal points)	HW
Caudal peduncle height	CPH
Length from the anus to the fork	AFL
Meristic	
Second dorsal fin rays	D
Anal fin rays	A
Left ventral fin rays	LV
Right ventral fin rays	RV
Left pectoral fin rays	LP
Right pectoral fin rays	RP
Caudal fin rays	C
Sub labial barbells	SB
Sub labial pores	SP
Tentacles on the left nostril	LN
Tentacles on the right nostril	RN
Teeth rows in inferior jaw	IJ
Teeth rows in superior jaw	SJ
Teeth rows in the left palate	LPL
Teeth rows in the right palate	RPL
Teeth rows in the vomer	VM

characters have been reported as independent of fish size (e.g. Tudela, 1999; Murta, 2000) and were therefore not standardised.

For each variable, differences between males and females were evaluated by *t* or Mann-Whitney tests (morphometric and meristic variables, respectively). Individuals were considered as multivariate observations in order to account for the joint effect of variables (Misra and Carscadden, 1987). A stepwise multivariate discriminant analysis was performed separately for morphometric and meristic data in order to identify the combinations of variables that best separate the samples of *H. didactylus* (Hair *et al.*, 1996). The effectiveness of the discriminant analysis was evaluated by means of the percentage of individuals correctly classified in the original sample and by a one-way ANOVA followed by *a posteriori* Bonferroni test procedures.

Morphological distances between samples were computed as Euclidean distances and their correlation with geographic distances was evaluated through a Mantel test.

All calculations were performed using SPSS (SPSS Inc.) and 0.05 as the level of significance.

Genetic analysis

Samples of muscle and liver tissue were hydrated and then disrupted using ultrasound prior to horizontal starch gel electrophoresis. The technical procedures for allozyme analysis followed Murphy *et al.* (1996) and the histochemical staining methods were adapted from Harris and Hopkinson (1976). Alleles were designated by their electrophoretic mobility relative to the anodal mobility of the most common allele, which was designated as 100. From the wide range of enzyme stains tested, only 10 (corresponding to 11 *loci*) showed variation between samples and were therefore assayed. This electrophoretic variation that was observed conformed to the simple Mendelian model of co-dominance and to the known quaternary structure of each enzyme (Pasteur *et al.*, 1985; Alayse, 1987).

The proportion of polymorphic *loci*, mean number of alleles per locus (MNA) and observed (H_O) and expected (H_E) heterozygizities were calculated in GENETIX 4.02 (Belkhir *et al.*, 1996-2001). A *locus* was considered polymorphic when the frequency of its most common allele did not exceed 0.95.

Wright's *F*-statistics and Nei's genetic distance (Nei, 1978) were used to analyse genetic differentiation and computed in GENETIX 4.02. Wright's *F*-statistics were estimated according to Weir and Cockerham (1984) and their significance was tested using permutations (1000 replicates). F_{IT}, F_{IS} and F_{ST} measure the departure from Hardy-Weinberg proportions at the level of the whole sample, individual samples and differentiation between samples, respectively (Wright, 1965). Confidence intervals (95%) for multilocus F_{ST} estimates were calculated by bootstrapping over loci. Deviations from Hardy-Weinberg equilibrium (HWE) were also tested according to the Marckov-chain method in GENEPOP 3.1 (Raymond and Rousset, 1995).

The model of isolation by distance (IBD) was evaluated by the correlation between genetic and geographical distances using the Mantel test (Mantel, 1967) as implemented in GENETIX 4.02. Geographic distances were computed as the shortest coastal distances between sites and genetic distances as $F_{ST}/(1-F_{ST})$. To compare morphological and molecular differentiation, the Mantel test was also performed in GENETIX 4.02 using morphological distances, defined as Euclidean distances, instead of geographic distances.

RESULTS

Morphological analysis

No statistical differences were found between males and females (t>0.282 or U>20854, P>0.05, for all tests), so sexes were pooled. After size adjustment calculations, all correlations between variables were lower than 0.6, and, as so, none of the variables were discarded prior to the discriminant analysis. The stepwise discriminant analyses performed on morphome-

TABLE 2. – Correlations between the morphometric and meristic variables included in the models and the discriminant functions (see Table 1 for variables' acronyms).

	Variable	Function 1	Function 2
Morphometric	DN2	0.713	0.181
	HW	0.521	-0.105
	CPH	0.253	-0.416
	IOD	0.346	0.414
	% VARIANCE	36.6	23.5
Meristic	LP	0.753	-0.285
	SB	0.722	-0.255
	RP	0.447	0.893
	C	-0.106	0.151
	% VARIANCE	59.6	20.4

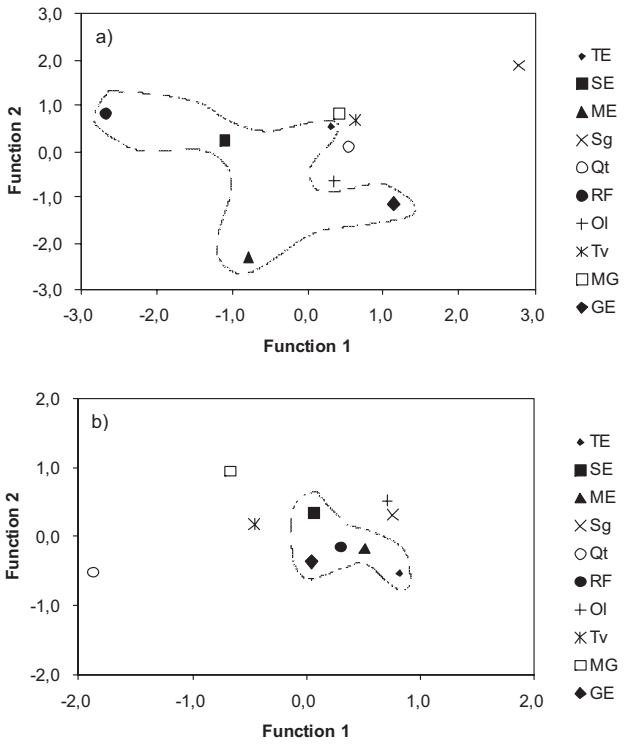

FIG. 2. – Plots of the centroids obtained from morphometric (a) and meristic (b) data analysis of *H. didactylus* samples, according to the first two discriminant functions. Estuarine samples are enclosed in the circle. TE, Tejo estuary; SE, Sado estuary; ME, Mira estuary; Sg, Sagres; Qt, Quarteira; RF, Ria Formosa estuary; Ol, Olhão; Tv, Tavira; MG, Monte Gordo; GE, Guadiana estuary.

tric and meristic data revealed that the first two discriminant functions explained 60.1% and 80.0% of total variance, respectively (Table 2). For the morphometric data, the first discriminant function was mainly correlated with DN2 (distance between the second pair of nostrils) and HW (head width), whereas the second function was mainly associated with CPH (caudal peduncle height) (negative correlation) and IOD (interorbital distance). For the meristic data, the variables presenting the highest correlations with the discriminant functions were LP (number of rays on the left pectoral fin) and SB (number of sublabial barbells) (function 1) and RP (number of rays on the right pectoral fin) (function 2) (Table 2).

The ordination diagrams obtained from the discriminant analysis of the morphometric data (Fig. 2a) revealed some differentiation between estuarine (dark symbols, left side) and coastal (light symbols, right side) samples along the first discriminant function. These differences were mainly related to the higher values of DN2 and HW presented by the coastal samples as indicated by the high and positive correlation coefficients of these variables with the first discriminant function (Table 2). The one-way ANOVA revealed significant differences between samples ($F>29.1$, $P<0.05$, in all tests) and the a posteriori Bonferroni test results showed that estuarine and coastal samples were always significantly different ($P<0.05$). For the meristic analysis it was not possible to identify a distinctive pattern of differentiation between estuarine and coastal samples in the discriminant analysis diagrams (Fig. 2b). However, estuarine samples showed a more homogeneous pattern, being distributed on the right side of the diagram, whereas coastal samples were distributed throughout the diagram. This pattern was probably related to the higher counts of LP, SB and RP in estuarine individuals, as indicated by the high and positive correlation coefficients of these variables with the first discriminant function (Table 2). One-way ANOVAs and Bonferroni *post-hoc* tests showed that these variables had statistically different values between the 10 samples ($F>1.3$, $P<0.05$, in all tests) and between coastal and estuarine samples ($P<0.05$), respectively.

According to the morphometric discriminant analysis, an average of 64.0% of the individuals were correctly classified and the highest successful classification was registered for individuals from Sagres (Sg) (100%) (Table 3). For the meristic characters, the average percentage of correctly classified individuals was considerably lower (31.9) and

TABLE 3. – Percentage of individuals of *H. didactylus* reallocated in each group in the validation of the discriminant analysis for the morphometric and meristic data. Rows are the original sample group and columns the reallocation group. Meristic data are in italics. GE, Guadiana estuary; MG, Monte Gordo; Tv, Tavira; Ol, Olhão; RF, Ria Formosa estuary; Qt, Quarteira; Sg, Sagres; ME, Mira estuary; SE, Sado estuary; TE, Tejo estuary.

	TE	SE	ME	Sg	Qt	RF	Ol	Tv	MG	GE
TE	38.5	7.7	0.0	0.0	3.8	7.7	9.6	21.2	3.8	7.7
	38.8	*4.1*	*12.2*	*18.4*	*0.0*	*0.0*	*10.2*	*8.2*	*0.0*	*8.2*
SE	7.7	61.5	3.8	0.0	7.7	5.8	5.8	3.8	3.8	0.0
	0.0	*60.0*	*0.0*	*4.0*	*4.0*	*0.0*	*12.0*	*8.0*	*12.0*	*0.0*
ME	2.9	2.9	91.4	0.0	0.0	0.0	0.0	0.0	0.0	2.9
	24.5	*3.8*	*13.2*	*20.8*	*5.7*	*0.0*	*7.5*	*17.0*	*3.8*	*3.8*
Sg	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
	9.6	*9.6*	*13.5*	*28.8*	*0.0*	*0.0*	*13.5*	*15.4*	*5.8*	*3.8*
Qt	7.1	12.5	1.8	3.6	55.4	0.0	3.6	5.4	1.8	8.9
	2.2	*0.0*	*2.2*	*0.0*	*67.4*	*0.0*	*0.0*	*10.9*	*13.0*	*4.3*
RF	0.0	13.0	6.5	0.0	0.0	76.1	0.0	4.3	0.0	0.0
	17.9	*8.9*	*14.3*	*12.5*	*5.4*	*0.0*	*10.7*	*10.7*	*8.9*	*10.7*
Ol	9.6	1.9	0.0	1.9	3.8	1.9	50.0	9.6	1.9	19.2
	18.8	*25.0*	*0.0*	*6.3*	*0.0*	*0.0*	*31.3*	*0.0*	*6.3*	*12.5*
Tv	11.3	3.8	1.9	0.0	3.8	3.8	9.4	62.3	1.9	1.9
	2.9	*2.9*	*8.6*	*5.7*	*17.1*	*0.0*	*2.9*	*40.0*	*11.4*	*8.6*
MG	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	96.0	0.0
	0.0	*9.6*	*3.8*	*3.8*	*9.6*	*0.0*	*13.5*	*5.8*	*50.0*	*3.8*
GE	4.1	0.0	4.1	0.0	10.2	0.0	12.2	6.1	2.0	61.2
	19.2	*5.8*	*7.7*	*5.8*	*15.4*	*0.0*	*17.3*	*7.7*	*7.7*	*13.5*

TABLE 4. – Enzyme systems, *loci* studied, separation technique, buffer system and tissue used in the genetic analysis of *H. didactylus* samples.

Protein	E. C.1	Locus 1	BUF. SYST.2	Tissue 3
Aspartate aminotransferase	2.6.1.1	AAT*	A	M
Acid phosphatase	3.1.3.2	ACP-1*	B	L
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH*	B	L
Glucose-6-phosphate isomerase	5.3.1.9	GPI-1*	A	M
		GPI-2*	A	M
Isocitrate dehydrogenase	1.1.1.42	IDH*	B	L
Malate dehydrogenase	1.1.1.37	MDH*	B	L
Malic enzyme	1.1.1.40	ME-1*	B	L
			B	L
Mannose-6-phosphate isomerase	5.3.1.8	MPI*	B	L
Phosphogluconate dehydrogenase	1.1.1.44	PGDH*	B	L
Phosphoglucomutase	5.4.2.2	PGM*	A	M

[1] According to Shaklee *et al*. (1990)
[2] Buffer system: A - tris-citrate-borate pH 8.7/8.2 (Pasteur *et al*., 1985); B - tris-citrate pH 8.0 (Pasteur *et al*., 1985)
[3] Tissue: L, liver; M, muscle

Quarteira (Qt) showed the highest successful classification (67.4%) (Table 3).

The Mantel test performed using the Euclidean distances showed that morphological distances were independent of geographic distances (Z=1563513.1, $P>0.05$).

Genetic analysis

Of the 11 *loci* assayed (Table 4), five (*AAT*, GPI-1*, GPI-2*, ME-1** and *PGM**) were monomorphic in all samples. A further two *loci* (*IDH** and *MDH**) were weakly polymorphic, with common allele frequencies higher than or equal to 0.95 in all the samples, whereas the remaining *loci* showed common allele frequencies lower than 0.95 in at least one sample (Table 5). Differences in the pattern of enzymatic systems between samples were also found, the most important being those found for *IDH**, *MDH** and *MPI**. *IDH** was polymorphic only for Tavira, Quarteira and Sado estuary samples, whereas *MDH** was polymorphic only in the Guadiana estuary, Quarteira and the Tejo estuary; *MPI** was polymorphic only in the Monte Gordo and Sado estuary samples but presented different alleles in each (MPI^{90} and MPI^{110}, respectively). The mean number of alleles per *locus* (MNA) varied between 1.00 and 1.55 and the mean expected heterozygosities (H_E) ranged from a minimum of <0.001 for several *loci* in different samples to a maximum of 0.62 for *G6PDH** in the Sado estuary (Table 6).

The extremely high and positive values of F_{IS} found for most *loci* in all samples indicated heterozygote deficiency. According to the permutations test only *IDH** showed non-significant F_{IS} values in all populations (Table 6). Most of the global depar-

TABLE 5. – Allelic frequencies for *H. didactylus* samples of the coastal areas analysed. GE, Guadiana estuary; MG, Monte Gordo; Tv, Tavira; Ol, Olhão; RF, Ria Formosa estuary; Qt, Quarteira; Sg, Sagres; ME, Mira estuary; SE, Sado estuary; TE, Tejo estuary. Sample size is indicated aside sampling site.

LOCUS	Alelle	GE(49)	MG(35)	TV(53)	OL(52)	Sample(n) RF(46)	QT(56)	SG(36)	ME(35)	SE(52)	TE(52)
ACP-1*	100	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	0.87	1.00
	110	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.13	0.00
G6PDH*	100	0.65	0.72	0.73	0.87	1.00	0.92	0.63	1.00	0.49	0.61
	110	0.25	0.28	0.11	0.13	0.00	0.05	0.37	0.00	0.17	0.20
	120	0.10	0.00	0.16	0.00	0.00	0.03	0.00	0.00	0.34	0.19
IDH*	80	0.00	0.00	0.03	0.00	0.00	0.02	0.00	0.00	0.03	0.00
	100	1.00	1.00	0.97	1.00	1.00	0.98	1.00	1.00	0.97	1.00
MDH*	90	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.02
	100	0.96	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	0.98
	110	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MPI*	90	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	100	1.00	0.96	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00
	110	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00
PGDH*	100	0.93	0.55	0.80	1.00	1.00	0.77	1.00	1.00	0.79	0.76
	110	0.07	0.45	0.20	0.00	0.00	0.23	0.00	0.00	0.21	0.23

TABLE 6. – Mean number of alleles across populations (MNA), observed (H_o) and expected (H_E) heterozygosities and departures from Hardy-Weinberg proportions (F_{IS}) for the six polymorphic *loci* within *H. didactylus* samples. Ria Formosa and Mira estuaries presented only monomorphic *loci* and are therefore not shown (* - $P<0.05$). GE, Guadiana estuary; MG, Monte Gordo; Tv, Tavira; Ol, Olhão; Qt, Quarteira; Sg, Sagres; SE, Sado estuary; TE, Tejo estuary.

		ACP-1*	G6PDH*	IDH*	MDH*	MPI*	PGDH*	MNA
	H_E	0.00	0.51	0.00	0.07	0.00	0.13	
GE	H_O	0.00	0.04	0.00	0.05	0.00	0.14	1.45
	F_{IS}	-	0.92*	-	0.33*	-	-0.04	
	H_E	0.00	0.40	0.00	0.00	0.08	0.49	
MG	H_O	0.00	0.00	0.00	0.00	0.04	0.22	1.27
	F_{IS}	-	1.00*	-	-	0.50*	0.57*	
	H_E	0.00	0.43	0.06	0.00	0.00	0.32	
Tv	H_O	0.00	0.04	0.07	0.00	0.00	0.14	1.36
	F_{IS}	-	0.92*	-0.02	-	-	0.60*	
	H_E	0.00	0.22	0.00	0.00	0.00	0.00	
Ol	H_O	0.00	0.00	0.00	0.00	0.00	0.00	1.09
	F_{IS}	-	1.00*	-	-	-	-	
	H_E	0.08	0.15	0.04	0.04	0.00	0.35	
Qt	H_O	0.00	0.00	0.04	0.02	0.00	0.03	1.55
	F_{IS}	1.00*	1.00*	-0.00	0.50*	-	0.93*	
	H_E	0.00	0.47	0.00	0.00	0.00	0.00	
Sg	H_O	0.00	0.00	0.00	0.00	0.00	0.00	1.09
	F_{IS}	-	1.00*	-	-	-	-	
	H_E	0.23	0.62	0.04	0.00	0.10	0.33	
SE	H_O	0.00	0.08	0.05	0.00	0.04	0.07	1.54
	F_{IS}	1.00*	0.88*	-0.00	-	0.66*	0.80*	
	H_E	0.00	0.55	0.00	0.04	0.00	0.36	
TE	H_O	0.00	0.05	0.00	0.02	0.00	0.05	1.36
	F_{IS}	-	0.92*	-	0.50*	-	0.86*	

ture from HWE (F_{IT}=0.839) was due to departures within samples (F_{IS}=0.832; F_{ST}=0.042). Overall exact tests of HWE using the Markov-chain method, under the null hypothesis of heterozygote deficiencies, were significant for all populations and *loci* ($P<0.05$). Using the same procedure, but only testing for HWE on populations, Olhão and Sagres were the only samples showing non-significant departures from equilibrium.

F_{ST} values were generally low (<0.100) but significant, the highest value registered being between Ria Formosa and its coastal area, Olhão (1.000) (Table 7, upper diagonal). Geographically close estuaries showed lower and significant F_{ST} values when compared to those obtained for distant ones: Tejo and Sado (0.001) vs. Tejo and Guadiana (0.007); Ria Formosa and Guadiana (0.381) vs. Ria Formosa and Tejo (0.455). Nei's genetic distances were also generally low (most values <0.010), the highest being registered between Ria Formosa and Monte Gordo (0.033) and Ria Formosa and the Sado estuary (0.031) (Table 7, lower diagonal). Although no consistent pattern was found, Nei's genetic distances were also generally lower between geographically close estuaries than between distant ones.

The Mantel test performed under 10000 permutations showed that genetic and geographic distances were independent, thus refuting the hypothesis of isolation by distance (Z=491.4, $P>0.05$). The Mantel test performed using genetic and morphological distances also showed that these were not correlated (Z=9030.0, $P>0.05$).

DISCUSSION

Morphological and genetic markers provide different but complementary information about population structure (Ihssen *et al.*, 1981) and have been widely used in population differentiation studies (e.g. Spanakis *et al.*, 1989; Alexandrino, 1996; Roldán *et al.*, 2000; Cabral *et al.*, 2003b, Pinheiro *et al.*, 2005).

According to the morphometric analysis, estuarine and coastal samples of *H. didactylus* showed some degree of differentiation along the Portuguese coast, the distance between the second pair of nostrils, head width, caudal peduncle height and interor-

TABLE 7. – Pairwise F_{ST} (upper diagonal) and Nei's genetic distance (below diagonal) between *H. didactylus* samples determined for all polymorphic *loci*. GE, Guadiana estuary; MG, Monte Gordo; Tv, Tavira; Ol, Olhão; RF, Ria Formosa; Qt, Quarteira; Sg, Sagres; ME, Mira estuary; SE, Sado estuary; TE, Tejo estuary. * indicates significant values.

	GE	MG	Tv	Ol	RF	Qt	Sg	ME	SE	TE
GE		0.102*	0.006*	0.003*	0.381*	0.074*	0.094*	0.120*	0.037	0.007*
MG	0.013		0.050	0.152*	0.212*	0.079*	0.054	0.000*	0.091*	0.021
Tv	0.002	0.006		0.015	0.516*	0.010*	0.106*	0.000*	0.029	0.024
Ol	0.003	0.019	0.004		1.000*	0.018	0.033	0.000*	0.097*	0.046
RF	0.012	0.033	0.010	0.001		0.592*	0.429*	0.000*	0.282*	0.455*
Qt	0.007	0.008	0.002	0.004	0.006		0.084*	0.639*	0.113*	0.054
Sg	0.000	0.017	0.007	0.004	0.016	0.012		0.000*	0.063*	0.178*
ME	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.818*	0.143*
SE	0.006	0.015	0.005	0.016	0.031	0.014	0.012	0.002		0.001*
TE	0.001	0.004	0.001	0.008	0.019	0.005	0.006	0.000	0.002	

bital distance being the most important characters in this distinction. Although showing a reduced discriminating power when compared to the morphometric traits, meristic counts of the number of rays on the pectoral and caudal fins and the number of sublabial barbells were also important factors in sample differentiation.

Allozymes revealed that the genetic variability of *H. didactylus* along the Portuguese coast was similar to that determined by Smith and Fujio (1982) over a large number of marine teleosts (mean heterozygosities of 0.006). The mean F_{ST} value found in the analysis of all *loci* (0.042) was lower than that reported by Ward *et al.* (1994) for fishes (0.14) but was in accordance with the low spatial genetic heterogeneity suggested by Gyllensten (1985) for marine teleosts. The Mantel test showed that genetic and geographic distances were not correlated, thus refuting the hypothesis of isolation by distance, and that morphological distances were independent of genetic ones. This pattern of low genetic divergence has been reported for other marine fishes (e.g. Gyllensten, 1985; Kinsey *et al.*, 1994; Chikhi *et al.*, 1998; Cabral *et al.*, 2003a, Pinheiro *et al.*, 2005).

A fine-scale genetic structure was detected between estuarine and coastal areas, as described for other species inhabiting both environments. Using allozyme markers, Ayvazian *et al.* (1994) detected genetic subdivision in the cobbler, *Cnidoglanis macrocephalus* (Valenciennes, 1840), a demersal sedentary fish inhabiting estuaries and nearshore marine areas in western and southern Australia. This species' reproductive biology is very similar to that observed in the Lusitanian toadfish, with eggs deposited in nests and males exhibiting parental care, therefore limiting larval dispersion. The authors detected a high genetic divergence between estuaries on a local and regional scale, suggesting low levels of gene flow between estuarine and marine populations. Chaplin *et al.* (1998) also reported genetically distinct assemblages of the black bream, *Acanthopagrus butcheri* Gomon *et al.*, 1994, in nine estuaries and one land-locked lake in western Australia, despite the low levels of allozymic variation. However, whereas in these species the greatest divergence was found between estuaries, the estuarine samples of *H. didactylus* showed low levels of genetic divergence. The high F_{ST} values between the Ria Formosa estuary and its adjacent coastal areas (Olhão and Quarteira) revealed an estuarine vs. coastal sample differentiation. A similar pattern was reported for *P. minutus* along its distribution range in Europe, with a clear distinction of the Venetian lagoons' samples towards the Mediterranean Sea and Atlantic Ocean (Stefanni *et al.*, 1996; 2003). The differentiation found in *H. didactylus* may have arisen from the operation of the barrier islands of Ria Formosa, which physically constrain the flux of individuals. Another plausible explanation is the "divergence-with-gene-flow" (Beheregaray and Sunnucks, 2001) induced by the sedentary behaviour and brood care of toadfish adults, which may contribute to a decrease in local genetic variability and subsequently increase differentiation among populations.

Many of the species found in a given estuary are likely to have resulted from distant immigration and undergone a recent founder effect or bottleneck (Bilton *et al.*, 2002). The genetic difference found between the Tejo and Sado estuaries, which are only a few kilometres apart, might reflect the fluctuation of the toadfish assemblage in the Tejo estuary, characterised by a great abundance period followed by an apparent complete disappearance of this species and a recent re-colonisation (Costa, 1993). Assuming that the heterozygote deficiency and high F_{IS} observed in this location are not the result of misinterpretations, the Whalund effect, inbreeding and directional selection of *loci* to which these individuals were subjected might explain such values. It would be expected that individuals from the nearest location, the Sado estuary, were the founders of the present day population in the Tejo estuary. The smallest F_{ST} value obtained between the Tejo estuary and the Sado estuary and their small Nei's genetic distance corroborate this hypothesis. However, the low values of these parameters obtained between the Guadiana estuary and the Tejo estuary, the furthest location, are not conclusive, so the process of such colonisation remains intriguing.

Studies performed with marine species whose populations inhabit separate coastal areas suggest an environmental basis for the high morphological differentiation. Tudela (1999) reported a high level of morphological heterogeneity in the European anchovy (*Engraulis encrasicolus* Linnaeus, 1758) from three sampling sites in the Mediterranean but, according to the electrophoretic analysis, they were genetically homogeneous. Similar results were found by Cabral *et al.* (2003b) for the Portuguese sole, *Synaptura lusitanica* Capello, 1868 inhabiting two locations about 250 km apart on the Portuguese

coast. Although phenotypic traits are highly variable and often have low heritabilities, reflecting substantial environmental influence (Lindsey, 1988), the overall low genetic differentiation found for *H. didactylus* should be investigated using more sensitive markers, such as mitochondrial DNA genes or microsatellites. Pampoulie *et al*. (2004) studied the genetic structure of *P. minutus* from the North Sea and, although no consistent differentiation was found using allozymes, microsatellite markers revealed a clear differentiation between estuarine and coastal areas samples. Due to their typical high allelic diversity, microsatellites increase the probability of detecting genetic differences among populations (Goudet *et al*., 1996), and may therefore, detect a genetic structure concordant with the high morphological differentiation of the Lusitanian toadfish on the Portuguese coast.

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REFERENCES

Alayse, J.P. – 1987. Le complexe *Solea lascaris*: mise en evidence par l'étude du polymorphisme enzymatique de deux espèces sympatriques en Mer d'Iroise. *Biochem. Syst. Ecol.*, 15: 204–273.

Alexandrino, P. – 1996. Genetic and morphological differentiation among some Portuguese populations of allis shad *Alosa alosa* (L., 1758) and twaite shad Alosa fallax (Lacépède, 1803). *Pub. Espec. Inst. Esp. Oceanog.*, 21: 15-24.

Avise, J.C., C.A. Reeb and N.C. Saunders. – 1987. Geographic population structure and species differences in mitochondrial DNA of mouthbrooding marine catfishes (Ariidae) and demersal spawning toadfishes (Batrachoididae). *Evolution*, 41: 991-1002.

Ayvazian, S.G., M.S. Johnson and D.J. McGlashan. – 1994. High levels of genetic subdivision of marine and estuarine populations of the estuarine catfish *Cnidoglanis macrocephalus* (Plotosidae) in southwest Australia. *Mar. Biol.*, 118: 25-31.

Beheregaray, L.B. and P. Sunnucks. – 2001. Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*. *Mol. Ecol.*, 10: 2849-2866.

Belkhir, K., P. Borsa, L. Chikhi, N. Rafauste and F. Bonhomme. – 1996-2001. GENETIX 4.02, logiciel sous Windows TM pour la génétique des populations. Université de Montpellier II, Montpellier.

Bilton, D.T., J. Paula and J.D.D. Bishop. – 2002. Dispersal, genetic differentiation and speciation in estuarine organisms. *Est. Coast. Shelf Sci.*, 55: 937-952.

Cabral, H.N., F. Castro, D. Linhares and P. Alexandrino. – 2003a. Genetic differentiation of *Solea solea* (Linnaeus, 1758) and *Solea senegalensis* Kaup, 1858, (Pisces: Pleuronectiformes) from several estuarine systems of the Portuguese coast. *Sci. Mar.*, 67: 43-52.

Cabral, H.N., J.F. Marques, A.L. Rego, A.I. Catarino, J. Figueiredo and J. Garcia. – 2003b. Population differentiation of *Synaptura lusitanica* Capello, 1868, along the Portuguese coast. *J. Sea Res.*, 50: 167-175.

Castilho, R. and B.J. McAndrew. – 1998. Population structure of seabass in Portugal: evidence from allozymes. *J. Fish Biol.*, 53: 1038-1049.

Chaplin, J.A., G.A. Baudains, H.S. Gill, R. McCulloch and I.C. Potter. – 1998. Are assemblages of black bream (*Acanthopagrus butcheri*) in different estuaries genetically distinct? *Int. J. Salt Lake Res.*, 6: 303-321.

Chikhi, L., F. Bonhomme and J.-F. Agnese. – 1998. Low genetic variability in a widely distributed and abundant clupeid species, *Sardinella aurita*. New empirical results and interpretations. *J. Fish Biol.*, 52: 861-878.

Costa, J.L. – 1993. Abundance and distribution of the toadfish *Halobatrachus didactylus* on the Mira estuary (Portugal): seasonal variations and ruling factors. *J. Fish Biol.*, 43 (Suppl. A): 330.

Goudet, J., S. Raymond, T. de Meeüs and F. Rousset. – 1996. Testing differentiation in diploid populations. *Genetics*, 144: 1933-1940.

Gyllensten, U. – 1985. The genetic structure of fish: differences in the intraspecific distribution of biochemical genetic variation between marine, anadromous and freshwater species. *J. Fish Biol.*, 26: 691-699.

Hair Jr., J.F., R. Anderson, R. Tatham and W. Black. – 1996. *Multivariate data analysis with readings*. Prentice Hall Incorporated, New Jersey.

Harris, H. and D.A. Hopkinson. – 1976. *Handbook of enzyme electrophoresis in human genetics*. North-Holland publishers, Amesterdam.

Humphries, J.M., F.L. Bookstein, B. Chernoff, G.R. Smith, R.L. Elder and S.G. Poss. – 1981. Multivariate discrimination by shape in relation to size. *Syst. Zool.*, 30: 291-308.

Hurlbut, T. and D. Clay. – 1998. Morphometric and meristic differences between shallow- and deep-water populations of White hake (*Urophycis tenuis*) in the southern Gulf of St Lawrence. *Can. J. Fish. Aquat. Sci.*, 55: 2274-2282.

Ihssen, P.E., D.O. Evans, W.J. Christie, J.A. Reckahn and R.L. DesJardine. – 1981. Life history, morphology, and electrophoretic characteristics of five allopatric stocks of Late whitefish (*Coregonus clupeaformis*) in the Great Lakes region. *Can. J. Fish. Aquat. Sci.*, 38: 1790-1807.

Kinsey, S.T., T. Orsoy, T.M. Bert and B. Mahmoudi. – 1994. Population structure of the Spanish sardine *Sardinella aurita*: natural morphological variation in a genetically homogeneous population. *Mar. Biol.*, 118: 309-317.

Lindsey, C.C. – 1988. Factors controlling meristic variation. *Fish Physiol.*, 11: 197-274.

Mantel, N. – 1967. The detection of disease clustering and a generalized regression approach. *Cancer Res.*, 27: 209-220.

Misra, R.K. and J.E. Carscadden. – 1987. A multivariate analysis of morphometrics to detect differences in populations of Capelin (*Mallotus villosus*). *J. Cons. Int. Explor. Mer*, 43: 99-106.

Murphy, R.W., J.W. Sites, D.G. Burth Jr. and C.H. Haufler. – 1996. Proteins: Isozyme electrophoresis. In: D.M. Hills, C. Moritz and B.K. Mable (eds.), *Molecular systematics,* pp. 51-120. Sinauer Associates, Massachussets.

Murta, A.G. – 2000. Morphological variation of Horse mackerel (*Trachurus trachurus*) in the Iberian and North African Atlantic: implications for stock identification. *ICES J. Mar. Sci.*, 57: 1240-1248.

Nei, M. – 1978. Estimation of the average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89: 583-590.

Nielsen, E.E., P.H. Nielsen, D. Meldrup and M.M. Hansen. – 2004. Genetic population structure of turbot (*Scophthalmus maximus* L.) supports the presence of multiple hybrid zones for marine fishes in the transition zone between the Baltic Sea and North Sea. *Mol. Ecol.*, 13: 585-595.

Pampoulie, C., E.S. Gysels, G.E. Maes, B. Hellemans, V. Leentjes, A.G. Jones and F.A.M. Volchaert. – 2004. Evidence for fine-scale genetic structure and estuarine colonisation in a potential high gene flow marine goby (*Pomatischistus minutos*). *Heredity*, 92: 434-445.

Pasteur, N., M. Autem, P. Pichot and M. Goucha. – 1985. Structure génétique de la sole (*Solea vulgaris* Quensel, 1806; Téléostéens, Soléidés): premier catalogue de polymorphismes

biochemiques acessibles par l'electrophorèse en gel d'amidon. *Rev. Trav. Inst. Pêches Mar.,* 47: 37-54.
Pinheiro, A., C.M. Teixeira, A.L. Rego, J.F. Marques and H.N. Cabral. – 2005. Genetic and morphological variation of *Solea lascaris* (Risso, 1810) along the Portuguese coast. *Fish. Res.*, 73: 67-78.
Raymond, M. and F. Rousset. – 1995. GENEPOP: population genetics software for exact tests and ecumenism. *J. Heredity*, 86: 248-249.
Ricker, W.E. – 1973. Linear regressions in fishery research. *J. Fish. Res. Board Can.*, 30: 409-434.
Roldán, M.I., R.G. Perrotta, M. Cortey and C. Pla. – 2000. Molecular and morphologic approaches to discrimination of variability patterns in Chub mackerel, *Scomber japonicus*. *J. Exp. Mar. Biol. Ecol.*, 253: 63-74.
Roux, C. – 1986. Batrachoididae. In: P.J.P. Whitehead, M.L. Bauchot, J.C. Hureau, J. Nielsen and E. Tortonese. (eds.) *Fishes of the North-eastern Atlantic and Mediterranean*, vol. III, 1360-1361. UNESCO, Paris.
Santos, M.E., T. Modesto, R.J. Matos, M.S. Grober, R.F. Oliveira and A. Canário. – 2000. Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. *Bioacoustics*, 10: 309-321.
Shaklee, J.B., F.W. Allendorf, D.C. Moritz and G.S. Whitt. – 1990. Gene nomenclature for protein-coding loci in fish. *Trans. Amer. Fish. Soc.*, 119: 2-15.
Smith, P.J. and Y. Fujio. – 1982. Genetic variation in marine teleosts: high variability in habitat specialists and low variability in habitat generalists. *Mar. Biol.*, 69: 7-20.
Sobral, D. and J. Gomes. – 1997. *Peixes litorais. Estuário do Sado*. ICN, Lisboa.
Spanakis, E., N. Tsimenides and E. Zouros. – 1989. Genetic differences between populations of sardine, *Sardina pilchardus*, and anchovy, *Engraulis encrasicolus*, in the Aegean and Ionian seas. *J. Fish Biol.*, 35: 417-437.
Stefanni, S., P.J. Miller and P. Torricelli. – 1996. Studio comparativo di due popolazioni di *Pomatoschistus minutus* (Teleostei: Gobiidae) della laguna veneta e di Plymouth (UK). *Atti Conveg. Assoc. Ital. Ittiol. Acque Dolci (AIIAD)*, 4: 385-390.
Stefanni, S., E.S. Gysells, F.A.M. Volckaert and P.J. Miller. – 2003. Allozyme variation and genentic divergence in the sand goby, *Pomatoschistus minutus* (Teleostei: Gobiidae). *J. Mar. Biol. Assoc. U.K.*, 83: 1143-1149.
Tudela, S. – 1999. Morphological variability in a Mediterranean, genetically homogeneous population of the European anchovy, *Engraulis encrasicolus*. *Fish. Res.*, 42: 229-243.
Ward, R.D., M. Woodwark and O.F. Skibinski. – 1994. A comparison of genetic diversity levels in marine, freshwater and anadromous fishes. *J. Fish Biol.*, 44: 213-232.
Weir, B.S. and C.C. Cockerham. – 1984. Estimating F-statistics for the analysis of population structure. *Evolution*, 38: 1358-1370.
Wright, S. – 1965. The interpretation of population structure by *F*-statistics with special regard to systems of mating. *Evolution*, 19: 395-420.

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