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Genetic differentiation of *Solea solea* (Linnaeus, 1758) and *Solea senegalensis* Kaup, 1858, (Pisces: Pleuronectiformes) from several estuarine systems of the Portuguese coast*

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SUMMARY: The genetic differentiation of *Solea solea* and *Solea senegalensis* from several estuarine systems along the Portuguese coast was studied. Nine polymorphic isozyme *loci* (*ACP-1**, *ACP-2**, *GPI-1**, *GPI-2**, *sMDH**, *ME-1**, *ME-2**, *MPI** and *PGM**) were analysed using starch gel electrophoresis and isoelectric focusing. Differentiation between the species was high (mean average Nei distance of 0.93). The most efficient *loci* in the diagnosis of the two species were *ACP-1**, *ME-2** and *GPI-2**. *S. solea* showed a higher genetic diversity than *S. senegalensis*. Within each species a low genetic differentiation between the samples analysed was found. Although with a low magnitude the interpopulational genetic differentiation of *S. solea* was higher than that of *S. senegalensis*. This could probably be explained by some particularities of the life cycles of these species, namely the more extended period of occurrence of larval stages of *S. senegalensis* in the plankton. Although no clear evidence about the population structure model emerged from the analysis of several Atlantic and Mediterranean populations of *S. solea*, the significant correlations obtained between genetic and geographical distances support an isolation by distance model.

Key words: genetic structure, population genetics, allozyme, *Solea*, Portugal.

RESUMEN: DIFERENCIACIÓN GENÉTICA DE *SOLEA SOLEA* (LINNEUS, 1758) Y *SOLEA SENEGALENSIS* KAUP, 1858), (PISCES: PLEURONECTIFORMES) EN VARIOS SISTEMAS ESTUÁRICOS DE LA COSTA PORTUGUESA. – Se estudió la diferenciación genética de *Solea solea* y *Solea senegalensis* en varios sistemas estuáricos a lo largo de la costa de Portugal. Se analizaron nueve *loci* de isozimas polimórficos (*ACP-1*, ACP-2*, GPI-1*, GPI-2*, sMDH*, ME-1*, ME-2*, MPI** and *PGM**) usando electroforesis en gel de almidón y enfoque isoeléctrico. La diferenciación entre especies fue alta (media de la distancia Nei 0.93). El *loci* más eficiente en la diagnosis de las dos especies fue *ACP-1*, ME-2** and *GPI-2**. *S. solea* presentó una mayor diversidad genética que *S. senegalensis*. Se encontró una baja diferenciación genética entre muestras analizadas dentro de una misma especie. Aunque con una baja magnitud, la diferenciación genética entre poblaciones de *S. solea* fue más alta que la de *S. senegalensis.* Esto podría explicarse, probablemente, por algunas particularidades de los ciclos de vida de estas especies, como el más prolongado periodo de aparición de los estadios larvarios de *S. senegalensis* en el plancton. Aunque no hay una evidencia clara sobre el modelo de estructura de la población de *S. solea,* las correlaciones significativas obtenidas apoyan un aislamiento por un modelo de distancia.

Palabras clave: estructura genética, genética de poblaciones, allozymas, *Solea*, Portugal.

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INTRODUCTION

Estuarine fish communities comprise species with different life-history patterns. A particularly important component of these communities is represented by marine species that use estuarine systems as nursery areas, in order to benefit from the high food availability and the existence of few predators (Haedrich, 1983; McLusky, 1989).

European sole, *Solea solea* (Linnaeus, 1758) and Senegalese sole, *Solea senegalensis* Kaup, 1858, are marine species with this kind of life cycle. These two flatfish species have a very similar morphology and a sympatric distribution from North Africa and the western Mediterranean up to the Bay of Biscay (Quéro *et al*., 1986). While ecological information concerning *S. senegalensis* is scarce and refers almost exclusively to the Portuguese coast (e.g. Andrade, 1989; Costa and Bruxelas, 1989; Cabral and Costa, 1999), substantial knowledge has been gained on *S. solea*, mainly in north European estuaries and coastal areas. The adult populations of *S. solea* are located on the continental shelf, where spawning takes place at water depths ranging from 40 to 100 m (Koutsikopoulos *et al.*, 1989; Koutsikopoulos and Lacroix, 1992). Larvae and juveniles tend to migrate to coastal areas by both passive and active transport processes that are not yet completely understood (Marchand and Masson, 1989, Koutsikopoulos and Lacroix 1992; Amara *et al*., 1994, 1998). Juveniles of *S. solea* concentrate in estuaries and in bays for a period of about two years, after which they start to migrate off coastal areas (Koutsikopoulos *et al*., 1989).

As for other marine fish species, the main feature of the life cycle of *S. solea* and *S. senegalensis* is a division into a juvenile phase that is predominantly estuarine and an adult phase that is marine. This life-history pattern may have an impact on the structuring of offshore adult populations, particularly on the genetic differentiation, since a strong association between a particular spawning and nursery area can be expected.

Although several studies on the genetics of *Solea* spp. have been carried out (e.g. Quignard *et al*., 1984; Pasteur *et al*., 1985; Goucha *et al.*, 1987; She *et al.*, 1987), only a few were focused on population genetics (*e.g.* Koutolas *et al*., 1995; Exadactylos *et al*., 1998). According to Koutolas *et al*. (1995), some features of the life cycle of *S. solea* may induce a low genetic flux; these include the homing behaviour to spawning grounds and physical barriers to larval dispersion. Other features might, however, result in "large scale" genetic exchanges, conferred by the long duration of the larval period and a high individual fecundity.

The present work aims to study the genetic differentiation between *S. solea* and *S. senegalensis* along the Portuguese coast based on isozyme analysis. This geographical area is particularly of interest since almost no other studies have been done and this is the principal zone in the Atlantic where these species are sympatric. Furthermore, the geomorphological particularities of the Portuguease coast, e.g. a narrow continental shelf divided by deep canyons, could induce a different population substructuring pattern compared to north European areas, which are much shallower. The results obtained for the Portuguese coast may also provide a better understanding of the genetic differentiation of these species a broader geographical scale, especially with regard to the differences between north European and Mediterranean populations reported by several authors (e.g. Koutolas *et al.*, 1995; Exadactylos *et al.*, 1998).

MATERIAL AND METHODS

Samples were collected between July and August 1997 in four estuarine systems of the Portuguese coast: Ria de Aveiro, Tagus and Mira estuaries and Ria Formosa (Fig. 1). Beam trawls and trap nets were used to catch the fish. All specimens caught were less than two years old. Due to their differential pattern of abundance, few individuals of *S. senegalensis* were captured in Mira estuary (and thus they were not considered for the genetic analysis) and none of *S. solea* in Ria Formosa.

Liver, muscle and blood tissue samples were taken immediately after capture and stored at –80ºC until electrophoretic analysis. Liver and muscle cells were disrupted by use of ultrasound after adding distilled water. The tissue enzymes were analysed by horizontal starch gel electrophoresis (SGE) or isoelectric focusing (IEF). IEF was used since for some of the markers the interpretations of the patterns in the gels were not satisfactory when SGE was used. The technical procedures of these methods are described in Murphy *et al*. (1996) and Alexandrino *et al*. (1996). Histochemical staining methods were adapted from Harris and Hopkinson (1976). Among the 24 loci examined (Table 1), only a sub-set of nine loci was used in the analysis, since when a cer-

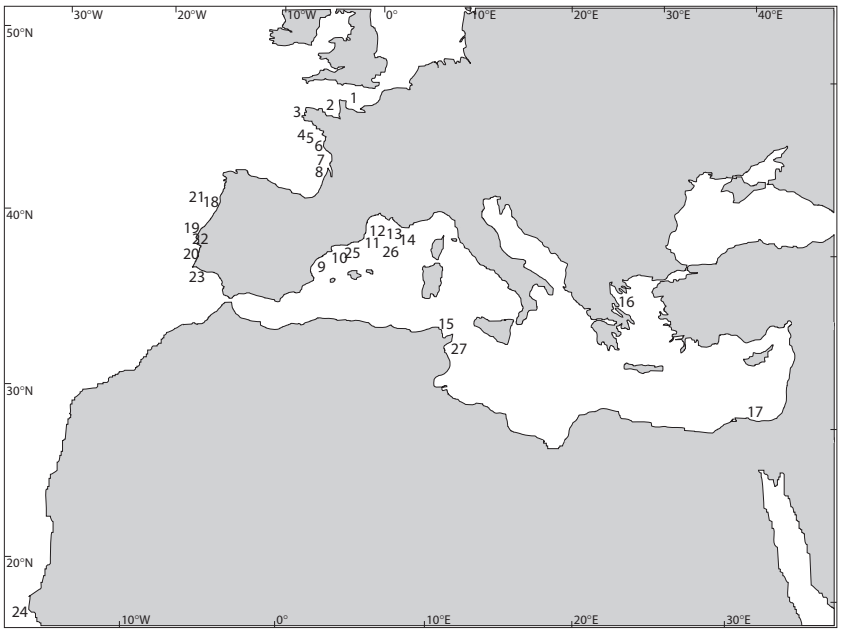

FIG. 1. – Atlantic and Mediterranean samples for which the data on the common genetic markers were analysed (Samples 1 to 17: *S. solea*, Koutolas *et al.* (1995) data; 18 to 20: *S. solea*, this study (18: Ria de Aveiro; 19: Tagus estuary; 20: Mira estuary); 21 to 23: *S. senegalensis*, this study (21: Ria de Aveiro; 22: Tagus estuary; 23: Ria Formosa); 24 and 25: *S. senegalensis*, Goucha *et al.* (1987) data; 26 and 27: *S. aegyptiaca*, Goucha *et al.* (1987) data).

TABLE 1. – Enzyme systems, *loci* studied, separation technique, buffer system and tissue.

Protein	E. C.[1]	*Locus* [1, 2]	Sep. tec./ Buf. syst.[3]	Tissue [4]
Aspartate aminotransferase	2.6.1.1	*sAAT-1** [(a)]		
		*sAAT-2** [(a)]	SGE/C, D, E	L, M
		*sAAT-3** [(a)]		
Acid phosphatase	3.1.3.2	*ACP-1**	SGE/B	L
		*ACP-2**	IEF	
Adenylate kinase	2.7.4.3	*AK** [(a)]	SGE /D	L, M
Creatine kinase	2.7.3.2	*CK** [(a)]	SGE /D	L, M
Esterase	3.1.1.-	*EST-1** [(a)]		
		*EST-2** [(a)]	SGE/C	L, M
		*EST-3** [(a)]		
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3PDH** [(b)]	SGE/C	L, M
Glucose-6-phosphate isomerase	5.3.1.9	*GPI-1**	SGE/A	M
		*GPI-2**		
Hemoglobine	-	*HB** [(b)]	IEF	B
L-Iditol-dehydrogenase	1.1.1.14	*sIDDH** [(b)]	SGE/D	L, M
L-Lactate dehydrogenase	1.1.1.27	*LDH-2** [(b)]	SGE/A	L, M
		*LDH-3** [(b)]		
Malate dehydrogenase	1.1.1.37	*sMDH**	SGE/C	M
Malic enzyme ($NADP^+$)	1.1.1.40	*ME-1**	SGE/C	M
		*ME-2**		
Mannose-6-phosphate isomerase	5.3.1.8	*MPI**	SGE/C	L
Phosphogluconate dehydrogenase	1.1.1.44	*PGDH** [(b)]	SGE/C	L, M
Phosphoglucomutase	5.4.2.2	*PGM**	SGE/A	M
Superoxide dismutase	1.15.1.1	*SOD-1** [(b)]	SGE/A	L, M

[1] according to Shaklee *et al.* (1990).
[2] (a) – Loci that could not be reliably scored; (b) – considered monomorphic in both species
[3] Separation Technique / Buffer system: SGE – Starch Gel Electrophoresis, IEF – Isoelectric focusing; A - tris-citrate-borate pH 8.7/8.2 (Pasteur et al., 1985); B - citrate-NaOH pH 6.0 (Ferrand and Amorim, 1990); C - tris-citrate pH 8.0 (Pasteur et al., 1985); D - tris-citrate pH 6.7 (Pasteur et al., 1985); E - tris-borate pH 8.0 (Siciliano and Shaw, 1976)
[4] Tissue : L – liver; M – muscle; B - blood.

tain enzymatic protein gave no satisfactory results or seemed to be monomorphic in both species it was omitted for further studies. Alleles were designated by their electrophoretic mobilities relative to the anodal mobility of the most common allele, which was designated as 100.

Allelic frequencies were calculated in BIOSYS-I (Swofford and Selander, 1985). The allelic frequen-

cies (inter-sample comparisons) and the genotypic frequencies (conformity to Hardy-Weinberg expectations) were compared by randomisation-based tests, using Fstat software (Goudet, 1998). *F*-statistics devised by Wright (1951) summarise population structure, explaining the proportion of the total genetic variation by differences within populations (F_{IS}) and between populations (F_{ST}). Both statistics were calculated by the method of Weir and Cockerham (1984), using Fstat software (Goudet, 1998). Cluster analysis was performed to evaluate the relationships among populations. Nei genetic distance (Nei, 1978) and the UPGMA clustering method were used and obtained from PHYLIP (Felsenstein, 1993). Comparisons with other populations of different geographical areas were made using Goucha *et al.* (1987) and Koutolas *et al.* (1995) data on the common genetic markers (*GPI-1**, *GPI-2**, *sMDH** and *PGM**). Despite the low number of polymorphic loci used, this analysis was made in order to produce some preliminary results on the genetic similarities of the Portuguese samples to those from other North Atlantic and Mediterranean areas. Several other studies also based a population analysis on

TABLE 2. – Allelic frequencies for *S. solea* and *S. senegalensis* samples of the estuarine systems analysed (*n:* sample size).

Locus	allele	*S. solea* R. Aveiro	*S. solea* Tagus	*S. solea* Mira	*S. senegalensis* R. Aveiro	*S. senegalensis* Tagus	*S. senegalensis* R. Formosa
*ACP-1**	*n*	*25*	*37*	*33*	*34*	*39*	*36*
	100	1.00	1.00	1.00	-	-	-
	110	-	-	-	1.00	1.00	1.00
*ACP-2**	*n*	*30*	*36*	*37*	*30*	*32*	*35*
	80	0.03	0.03	0.03	-	-	-
	100	0.28	0.38	0.35	-	-	-
	105	0.05	0.03	-	-	-	-
	115	0.32	0.28	0.36	-	-	-
	120	0.17	0.15	0.14	1.00	1.00	1.00
	130	0.15	0.13	0.12	-	-	-
*GPI-1**	*n*	*32*	*42*	*43*	*44*	*43*	*45*
	100	1.00	0.96	1.00	1.00	0.98	1.00
	115	-	0.04	-	-	0.02	-
*GPI-2**	*n*	*28*	*38*	*43*	*44*	*43*	*43*
	90	-	0.03	-	-	-	-
	95	-	-	0.02	-	-	-
	100	0.84	0.90	0.84	-	-	-
	105	-	-	-	-	-	0.01
	110	0.16	0.07	0.14	-	-	-
	120	-	-	-	0.99	1.00	0.99
	130	-	-	-	0.01	-	-
*sMDH**	*n*	*18*	*37*	*32*	*35*	*43*	*33*
	100	1.00	0.95	0.98	1.00	0.98	1.00
	110	-	0.05	0.02	-	0.02	-
*ME-1**	*n*	*28*	*39*	*17*	*34*	*41*	*21*
	90	-	-	0.03	0.39	0.39	0.35
	95	0.07	0.03	0.09	0.59	0.57	0.58
	100	0.93	0.97	0.88	0.02	0.04	0.07
*ME-2**	*n*	*16*	*23*	*23*	*41*	*41*	*45*
	100	1.00	1.00	1.00	-	-	-
	110	-	-	-	1.00	1.00	1.00
*MPI**	*n*	*32*	*37*	*38*	*44*	*43*	*38*
	85	0.14	0.32	0.15	0.01	-	-
	95	-	0.02	-	0.97	1.00	1.00
	100	0.70	0.61	0.85	0.02	-	-
	105	0.13	-	-	-	-	-
	115	0.03	0.03	-	-	-	-
	120	-	0.02	-	-	-	-
*PGM**	*n*	*30*	*40*	*43*	*44*	*43*	*45*
	80	0.03	-	-	0.01	0.03	-
	100	0.89	0.95	0.95	0.99	0.88	0.97
	115	0.08	0.05	0.05	-	0.09	0.03

few loci, some not particularly highly polymorphic (e.g. Blanquer *et al.*, 1992; Castilho and McAndrew, 1998; Chaplin *et al.*, 1998). For this analysis allele relative mobility assignments were made. Data of 20 populations of *S. solea* (11 from the Atlantic coast, i.e. 8 from North Europe and 3 from Portugal, and 9 from the Mediterranean), 5 of *S. senegalensis* (4 from the Atlantic coast, i.e. 3 from Portugal and 1 from North Africa, and 1 from the Mediterranean) and 2 of *S. aegyptiaca* (from the Mediterranean) were used in this analysis (Fig. 1). Nei genetic distance and the UPGMA clustering method were also used in this analysis and obtained from PHYLIP (Felsenstein, 1993). Pairwise genetic and geographic distance calculations (taking into account the curvature of coastline) were made between these 20 populations of *S. solea*, in order to evaluate the existence of random mating and gene flow. The Mantel test, performed in BIOMstat (Rohlf and Slice, 1995), was used to estimate the association between these two matrices.

TABLE 3. – F_{ST}, F_{IS} and F_{IT} estimates per locus for the *S. solea* and *S. senegalensis* samples analysed.

	S. solea		
Locus	F_{ST}	F_{IS}	F_{IT}
*ACP-2**	-0.007	0.022	0.015
*GPI-1**	0.022	-0.024	-0.001
*GPI-2**	0.005	0.016	0.020
*sMDH**	0.011	-0.031	-0.020
*ME-1**	-0.002	0.686	0.686
*MPI**	0.058	0.065	0.119
*PGM**	0.004	-0.061	-0.056
Total	0.013	0.067	0.079
	S. senegalensis		
Locus	F_{ST}	F_{IS}	F_{IT}
*GPI-1**	0.012	-0.012	0.000
*GPI-2**	-0.003	0.000	-0.003
*sMDH**	0.019	-0.0022	-0.003
*ME-1**	-0.014	0.142	0.130
*MPI**	0.014	-0.015	-0.001
*PGM**	0.047	-0.079	-0.028
Total	-0.002	0.091	0.090

RESULTS

Nine of the surveyed *loci* were polymorphic (Table 2), *ACP-1** and *MPI** being described for the first time for these species. *ACP-1**, *GPI-2** and *ME-2** were diagnostic *loci* for *S. solea* and *S. senegalensis*, each one presenting specific alleles for each species.

Six putative *loci* of *ACP-2** were identified in *S. solea*, while for *S. senegalensis* only one was found. *ACP-2*100*, *ACP-2*115*, *ACP-2*120* and *ACP-2*130* were common alleles in *S. solea* samples. For *S. senegalensis* only *ACP-2*120* was detected.

The pattern observed for *GPI-1** was similar for both species, two putative alleles being recorded. For *GPI-2**, the alleles identified in *S. solea* were different from those of *S. senegalensis*. *GPI-2*100* showed the highest frequencies for *S. solea* while *GPI-2*120* did for *S. senegalensis*. All the other variants, except *GPI-2*110* in *S. solea*, were detected in low frequency.

A similar frequency distribution was noticed for *sMDH** in both species, *sMDH*110* being a low frequency allele. Concerning *ME-1**, *ME-1*100* was the most common allele in *S. solea*, with frequencies from 0.88 to 0.97. Although with lower values, *ME-1*95* was also a common allele (frequency values from 0.03 to 0.09). This allele was the most represented in *S. senegalensis*, with values ranging from 0.57 to 0.59. For the latter species, *ME-1*90* also showed a high frequency (from 0.35 to 0.39), while *ME-1*100* was rare.

For *MPI**, the allelic diversity recorded in *S. solea* was higher than that obtained for *S. senegalensis*. For the first species, *MPI*100* was the most common allele (with frequency values from 0.61 to 0.85). The majority of the other putative alleles were less common and some were limited to certain populations. For *S. senegalensis MPI*95* showed extremely high frequencies (between 0.97 and 1.00).

Finally, the results obtained for *PGM** were similar in both species, *PGM*100* being the most common allele, with frequency values from 0.88 to 0.99.

A significant deviation from Hardy-Weinberg equilibrium was observed only in one test corresponding to a deficiency in expected heterozygote proportion: *ME-1** ($p<0.05$ in randomisations tests, following the adjustments for multiple simultaneous tests).

The values of mean heterozygosity determined over 16 *loci* (over the 9 polymorphic and 7 monomorphic loci that were considered, see Table 1) for *S. solea* and *S. senegalensis* were 0.121 and 0.048 respectively. The analysis of the F-statistics determined for both species shows that only 1.3 and 0.2% (respectively for *S. solea* and *S. senegalensis*) are due to differences among populations (Table 3). The randomisations tests used to assess population differentiation showed no significant differences

TABLE 4. – Estimates of Nei genetic distances among the samples of *S. solea* and *S. senegalensis* analysed along the Portuguese coast.

		S. solea			*S. senegalensis*		
		R. Aveiro	Tagus	Mira	R. Aveiro	Tagus	R. Formosa
S. solea							
	R. Aveiro	-					
	Tagus	0.0070	-				
	Mira	0.0041	0.0084	-			
S. senegalensis							
	R. Aveiro	0.9088	0.9310	0.9144	-		
	Tagus	0.9375	0.9618	0.9472	0.0014	-	
	R. Formosa	0.9031	0.9248	0.9116	0.0004	0.0010	

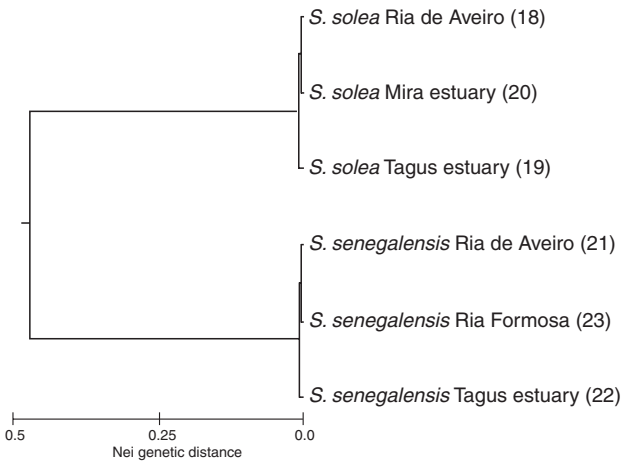

FIG. 2. – UPGMA dendrogram of Nei genetic distances (Table 4) obtained from the Portuguese samples of *S. solea* and *S. senegalensis* (numbers between brackets are relative to the sites presented in Fig. 1).

between allelic frequencies for both species ($p<0.05$, considering all *loci*).

Nei genetic distance (Nei, 1978) values between the two species were high (mean value 0.93), while within populations of each species they were extremely low (mean value less than 0.007), the estimates for *S. senegalensis* being lower (Table 4). The dendrogram based on Nei genetic distance (Nei, 1978) between the Portuguese samples of *S. solea* and *S. senegalensis* indicated the genetic discreteness of the two species, and it was noticed that for both species the Tagus samples were grouped separately from the other sampling sites (Fig. 2).

The dendrogram that resulted from the cluster analysis using several European and African populations of *S. solea*, *S. senegalensis* and *S. aegyptiaca* showed that these three species were well differenti-

TABLE 5. – Estimates of Nei genetic distances among 27 Atlantic and Mediterranean samples of

	1	2	3	4	5	6	7	8	9	10	11	12
2	0.0008											
3	0.0017	0.0025										
4	0.0009	0.0008	0.0026									
5	0.0007	0.0013	0.0011	0.0011								
6	0.0012	0.0013	0.0014	0.0012	0.0005							
7	0.0008	0.0006	0.0013	0.0007	0.0004	0.0003						
8	0.0010	0.0011	0.0031	0.0012	0.0016	0.0026	0.0015					
9	0.0049	0.0036	0.0089	0.0029	0.0067	0.0071	0.0053	0.0031				
10	0.0039	0.0021	0.0058	0.0019	0.0044	0.0037	0.0025	0.0033	0.0023			
11	0.0033	0.0024	0.0067	0.0016	0.0046	0.0049	0.0036	0.0020	0.0004	0.0018		
12	0.0026	0.0013	0.0052	0.0013	0.0036	0.0036	0.0022	0.0015	0.0010	0.0008	0.0007	
13	0.0017	0.0013	0.0045	0.0013	0.0028	0.0035	0.0023	0.0007	0.0014	0.0026	0.0008	0.0009
14	0.0013	0.0013	0.0039	0.0013	0.0025	0.0034	0.0020	0.0005	0.0020	0.0026	0.0013	0.0009
15	0.0018	0.0020	0.0049	0.0014	0.0032	0.0042	0.0030	0.0008	0.0016	0.0037	0.0011	0.0014
16	0.0125	0.0084	0.0156	0.0086	0.0138	0.0123	0.0102	0.0101	0.0044	0.0029	0.0053	0.0041
17	0.0140	0.0109	0.0176	0.0107	0.0148	0.0133	0.0119	0.0113	0.0085	0.0075	0.0081	0.0074
18	0.0037	0.0047	0.0039	0.0048	0.0030	0.0034	0.0034	0.0042	0.0114	0.0088	0.0085	0.0072
19	0.0023	0.0015	0.0045	0.0017	0.0031	0.0035	0.0022	0.0012	0.0024	0.0024	0.0017	0.0010
20	0.0012	0.0020	0.0017	0.0022	0.0009	0.0014	0.0012	0.0020	0.0078	0.0058	0.0057	0.0044
21	0.2575	0.2639	0.2271	0.2634	0.2503	0.2480	0.2507	0.2682	0.2916	0.2699	0.2845	0.2774
22	0.2777	0.2846	0.2461	0.2837	0.2700	0.2674	0.2703	0.2881	0.3130	0.2909	0.3050	0.2981
23	0.2584	0.2649	0.2281	0.2644	0.2512	0.2488	0.2515	0.2693	0.2927	0.7100	0.2856	0.2785
24	0.2277	0.2340	0.1993	0.2336	0.2206	0.2186	0.2213	0.2386	0.2615	0.2404	0.2547	0.2475
25	0.2899	0.2959	0.2583	0.2955	0.2831	0.2803	0.2834	0.3011	0.3211	0.3022	0.3142	0.3101
26	0.4792	0.4769	0.4415	0.4765	0.4709	0.4635	0.4650	0.4938	0.4981	0.4654	0.4962	0.4832
27	0.6347	0.6290	0.5906	0.6284	0.6259	0.6156	0.6167	0.6507	0.6476	0.6097	0.6476	0.6324

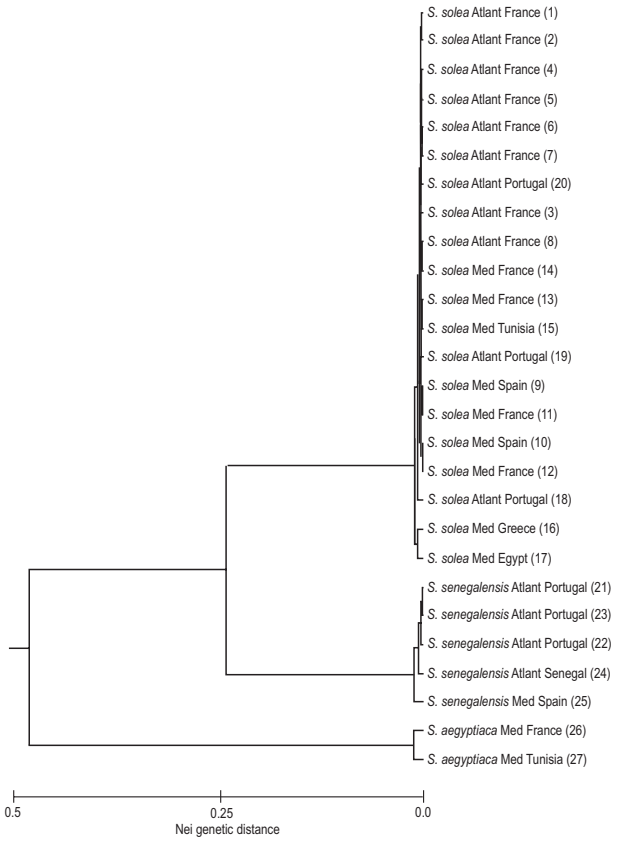

FIG. 3. – UPGMA dendrogram of Nei genetic distances (Table 5) obtained from the 27 Atlantic and Mediterranean samples of *S. solea*, *S. senegalensis* and *S. aegyptiaca* analysed (Atlant: Atlantic; Med: Mediterranean; numbers between brackets are relative to the sites presented in Fig. 1).

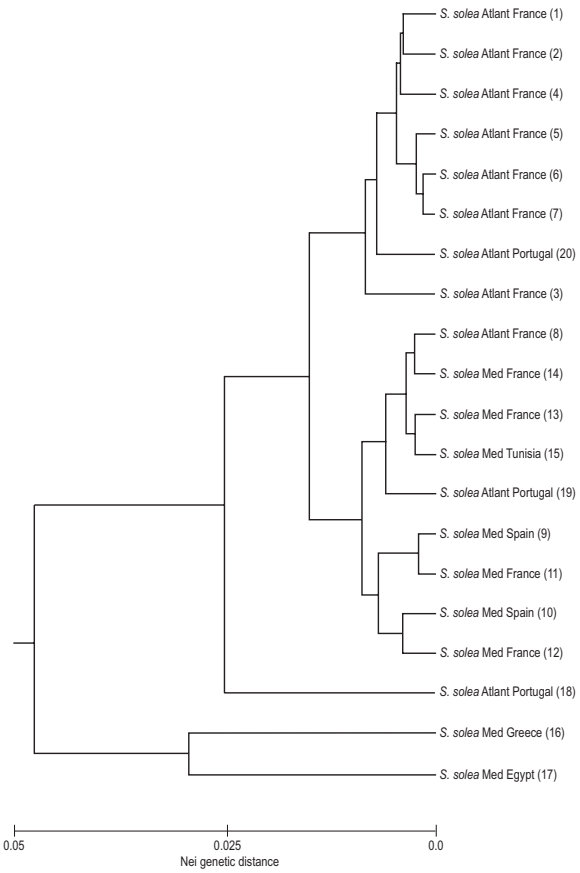

FIG. 4. – UPGMA dendrogram of Nei genetic distances (Table 5) obtained from the 20 Atlantic and Mediterranean samples of *S. solea* analysed (Atlant: Atlantic; Med: Mediterranean; numbers between brackets are relative to the sites presented in Figure 1).

S. solea, *S. senegalensis* and *S. aegyptiaca* analysed (numbers are relative to the sites presented in Fig. 1).

13	14	15	16	17	18	19	20	21	22	23	24	25	26
0.0006													
0.0005	0.0007												
0.0079	0.0082	0.0094											
0.0103	0.0100	0.0115	0.0064										
0.0065	0.0051	0.0069	0.0184	0.0120									
0.0011	0.0010	0.0016	0.0071	0.0065	0.0049								
0.0034	0.0028	0.0038	0.0150	0.0135	0.0020	0.0027							
0.2749	0.2727	0.2758	0.3013	0.3121	0.2579	0.2740	0.2495						
0.2956	0.2926	0.2963	0.3229	0.3246	0.2722	0.2922	0.2673	0.0025					
0.2759	0.2736	0.2768	0.3024	0.3112	0.2578	0.2745	0.2500	0.0003	0.0017				
0.2450	0.2430	0.2459	0.2712	0.2826	0.2290	0.2443	0.2199	0.0050	0.0096	0.0045			
0.3037	0.3061	0.3072	0.3346	0.3494	0.2959	0.3071	0.2833	0.0086	0.0119	0.0089	0.0156		
0.4953	0.4908	0.5011	0.4734	0.5171	0.4918	0.4934	0.4617	0.4120	0.4351	0.4137	0.3920	0.4075	
0.6502	0.6448	0.6580	0.6105	0.6657	0.6518	0.6478	0.6167	0.5759	0.6036	0.5782	0.5539	0.5606	0.0116

ated (Fig. 3). Despite the low values of genetic distance between *S. solea* populations, two main geographical clusters were identified: North Atlantic and Mediterranean populations (Fig. 4). The three Portuguese samples of *S. solea* were not consistently grouped in these two major clusters: the samples of Ria de Aveiro (18, in Figs. 3 and 4) appeared isolated from both North Atlantic and western Mediterranean sites, the samples of Mira estuary (20, in Figs. 3 and 4) were near the North Atlantic samples, and that of the Tagus estuary (19, in Figs. 3 and 4) was more closely related to the Mediterranean samples. The genetic distances obtained between the 20 *S. solea* samples considered were positively correlated with geographic distances (Mantel test: r=0.49, $p<0.01$).

DISCUSSION

As previously reported by other authors (e.g. Goucha *et al.*, 1987, Tinti and Piccinetti, 2000), *S. solea* and *S. senegalensis* are genetically well differentiated. The techniques developed in the present study revealed two new diagnostic *loci* (*ACP-1** and *ME-2**) for these two species and also described a new polymorphic *locus* (*MPI**). Several rare alleles from other *loci* may also be diagnostic, but the consistency of these markers is particularly dependent on sample size.

The genetic diversity values determined for the Portuguese samples of *S. solea* were lower than those reported for Northeastern Europe by Koutolas *et al*. (1995) and for Mediterranean areas by Goucha *et al*. (1987). Koutolas *et al*. (1995) reported a decrease in mean heterozygosity estimates from northern towards southern areas while Exadactylos *et al*. (1998) found an inverse relationship. The mean heterozygosity values determined by these authors are generally lower than those obtained for the Portuguese samples. However, these values are highly related to the number and type of the *loci* analysed and consequently limit comparisons between results from different studies. The values obtained for *S. senegalensis* Portuguese populations were within the range reported by other authors (e.g. Goucha *et al*., 1987). Although a lower number of studies are available for *S. senegalensis*, the genetic diversity determined for these species is lower than that reported for *S. solea*.

Although several aspects of the life-cycles of *S. solea* and *S. senegalensis* and certain oceanographic particularities of the Portuguese coast, namely the existence of several canyons that can act as barriers, could presumably favour genetic differentiation, the genetic differentiation between samples was very low. A general pattern of low genetic divergence among populations of marine fish species has been pointed out by several authors (e.g. Smith and Fujio, 1982; Gyllensten, 1985), who report that the level of differentiation (F_{ST}) expected for high mobile species usually ranges from 0 to 0.028 (Waples, 1987). Nevertheless, for some marine species with high dispersal capabilities, several studies have pointed out the existence of population differentiation. Lenfant and Planes (1996) outlined that some samples of *Diplodus sargus* (Linnaeus, 1758) showed genetic divergence from others, among several sites in the Mediterranean. Chaplin *et al.* (1998), in Australia, found considerable variation among samples of *Acanthopagrus butcheri* (Munro, 1949), a marine-estuarine opportunist species, even within a small geographical scale. For the Portuguese coast, Castilho *et al.* (1998) suggested that population structuring of *Dicentrarchus labrax* (Linnaeus, 1758) exists along the Portuguese coast, and reported some restriction to flow between the population of Algarve and all the other sites of the North.

The homogeneity among samples obtained in the present study, both for *S. solea* and *S. senegalensis*, de-emphasises the importance of estuarine systems as structuring forces of genetic differentiation. This could be due to a high genetic flux between populations at different stages of the life cycles, namely adult migration between spawning grounds and juvenile dispersion after the estuarine phase. However, several authors (e.g. Avise, 1994; Koutolas *et al*., 1995; Hillbish, 1996) suggest that larval period is probably the most important in this context, the genetic flux higher being with increasing duration of pelagic larval period. This could explain the lower values of genetic differentiation obtained for *S. senegalensis,* which on the Portuguese coast have a wider spawning period and consequently a more extended period of larval occurrence in the plankton (Dinis, 1986).

According to Koutolas *et al*. (1995), the patterns of genetic differentiation observed for *S. solea* in a broader geographical area suggest the existence of an isolation by distance populational structure, with large units being recognised in an east to west and north to south pattern of differentiation. High levels of gene flow between *S. solea* populations were reported by Exadactylos *et al*. (1998) in a study of several north European and

Mediterranean populations. However, these authors disagree with the isolation by distance model and suggest that the major populational differentiation shift occurs between Mediterranean and north European populations, which is corroborated by several Mediterranean biogeographers. All these studies focused mainly on northern Europe, south European Atlantic and Mediterranean population samples being very scarce.

No clear evidence about the population structure model emerged from the cluster analysis of several Atlantic and Mediterranean populations of *S. solea*. Although two major clusters were identified, i.e. North Atlantic and Mediterranean populations, there was not a complete segregation of populations according to geographical area, some Atlantic populations being grouped with Mediterranean ones. Furthermore, the samples from the Portuguese population were not consistently grouped in one of these main clusters. Nonetheless, the significant correlation obtained between genetic and geographical distances supports a non-random mating system and an isolation by distance model (e.g. King *et al.*, 2001; Wirth and Bernatchez, 2001).

The Portuguese coast may play a key role for studies focusing on the population structure of these flatfish species, since the Mediterranean influence is extended off Portugal (Fiúza, 1983). Further studies with standardised methodologies and a broader sampling area are needed for a better understanding of the genetic structure of these species.

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