Transactions of the American Fisheries Society 136:484–491, 2007

DOI: 10.1577/T06-030.1

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Genetic Relationships among Populations of the Senegalese Sole *Solea senegalensis* in the Southwestern Iberian Peninsula Detected by Mitochondrial DNA–Restriction Fragment Length Polymorphisms

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Abstract.—We assessed the population structure of the Senegalese sole *Solea senegalensis* in the southern Atlantic off the coast of the Iberian peninsula using restriction fragment length polymorphism analysis of three mitochondrial gene regions: 16S rRNA (620 base pairs [bp]), 12S rRNA (440 bp), and cytochrome *b* (380 bp). Fourteen composite haplotypes were detected among 109 adult fish from five natural populations. Haplotypic diversity values ranged between 0% and 18%, with the highest values in the Lisbon and Valdelagrana populations. A single composite haplotype predominated in most collections; two other haplotypes also occurred at low frequencies in three of the analyzed populations. The remaining 11 haplotyes were found only in single populations. A genetic differentiation index revealed significant differentiation between two groups (Lisbon and the other populations). However, from a conservative management perspective and based on the topology of the neighbor-joining consensus tree and bootstrap values (54–88), the possibility of three groups (Lisbon, Portimao, and the other populations) should be considered.

Genetic diversity within and among populations ensures the evolutionary potential of species in the long term and individual fitness in the short term (Lande 1988; Ferál 2002). Accordingly, knowledge of genetic structure and degree of gene flow among populations is needed to guide fishery management and conservation programs (Mustafa 1999). Thus, failure to detect population units can lead to local overfishing and ultimately to severe population declines (Palumbi and Wilson 1990).

The Senegalese sole *Solea senegalensis*, a common flatfish, is distributed off the eastern Atlantic coast from the northern part of Senegal to France and in the Mediterranean Sea from the Strait of Gibraltar to the coast of Tunisia (Dinis et al. 1999). Catch data for the Iberian peninsula indicate that density is high in the Gulf of Cadiz (Spain; Figure 1), less in Portuguese waters, and quite low in Cantabrian and Mediterranean waters (Rodriguez Martinez 1984; Rodriguez and Rodriguez 1980; Dinis et al. 1999). This species is gonochoric (i.e., well adapted to warm climates) and commonly raised in extensive earthen ponds along the southern coasts of Portugal and Spain. It is considered the most important commercial species in this region, with high economic value and high-quality flesh (Dinis et al. 1999; Imsland et al. 2003).

Solea senegalensis is an inshore species with a short larval development period and spawns in estuarine zones where temperatures range from 12°C to 17°C (Dinis et al. 1999). After day 19, when metamorphosis is complete, the larvae start their migration to inshore areas characterized by sandy bottoms (Dinis et al. 1999). As with other fish with pelagic development, physical factors (currents, depth, temperature, salinity boundaries, hydrodynamic eddies, and sea floor topography) and biological factors (predation and competition) exert a strong influence on the distribution and differentiation of its populations. However, it is of probably greater importance that the life history of this species is divided into an estuarine juvenile phase and a predominantly marine adult phase (Cabral et al. 2003). This life history pattern may have an impact on the structuring of adult populations, and particularly their genetic differentiation, since there is a strong association between particular spawning and nursery areas (Stepien 1999). Nonetheless, low levels of genetic differentiation have been reported for this species along the Portuguese coast (Cabral et al. 2003), and no genetic information is available for the Spanish populations. This lack of differentiation may reflect the low resolution level of the

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Received February 3, 2006; accepted October 6, 2006
Published online March 15, 2007

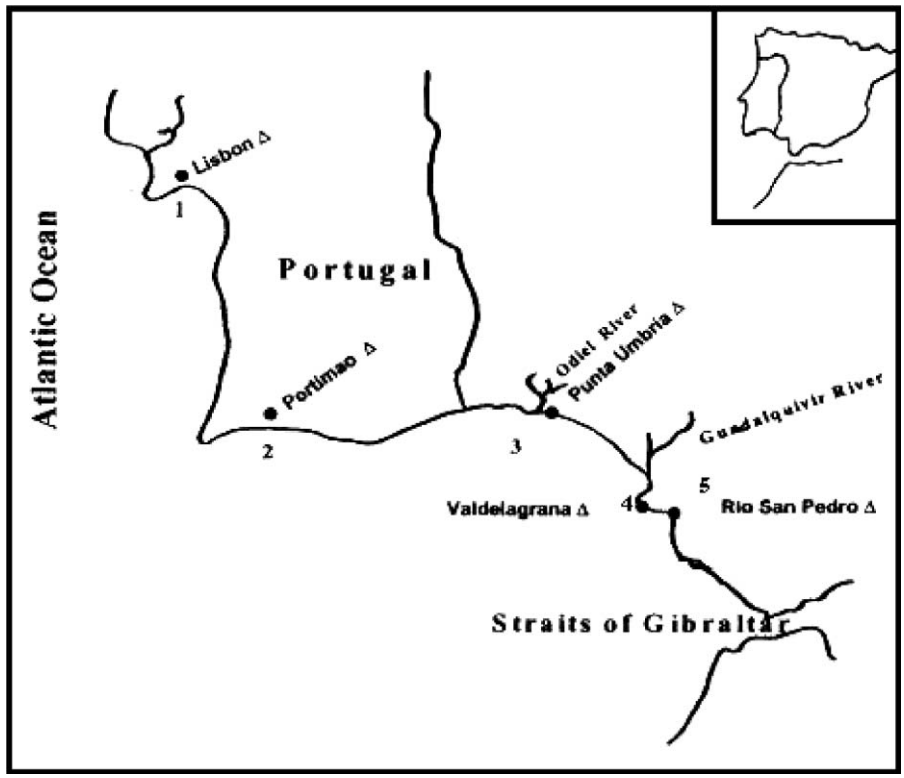

FIGURE 1.—Geographical locations of the five *Solea senegalensis* populations analyzed by means of mtDNA variations.

allozyme markers that have been used (Cabral et al. 2003) or a reduction in the larval retention and homing behavior of spawners due to specific physical and biological characteristics of the coast.

However, evidence of genetic differentiation has been noted in congeneric species with distributions overlapping those of *S. senegalensis*. Genetic differentiation consistent with isolation by distance was reported both in Dover sole *S. solea* (also known as sole *S. vulgaris*; Koutolas et al. 1995) and sand sole *S. lascaris* (Pinheiro et al. 2005). In contrast, other studies have shown differences between the Mediterranean and northern European populations of Dover sole, suggesting that local selection, genetic drift, or single-generation sampling effects are important in these areas (Exadactylos et al. 1998).

Mitochondrial DNA (mtDNA) has proven useful for evolutionary and population studies in eukaryotic organisms because of its intrinsic features and the ease with which informative DNA sequence data can be obtained by selective gene amplification using universally conserved primers (Kocher et al. 1989; Palumbi and Wilson 1990; Cunningham et al. 1992). The mtDNA genome is haploid and maternally inherited, resulting in a fourfold increase in the rate of genetic drift relative to nuclear DNA (Ovenden 1990). Variations of the entire mtDNA genome, individual genes, or restriction fragment length polymophisms (RFLPs) have been useful in characterizing taxa, establishing phylogenetic relationships, clarifying conspecific hybridizations, making maternal assignment tests, and assessing stocks in many fish species (Moritz 1994; Grant and Bowen 1998; Tinti et al. 2000). At the landscape genetics level, diversity within species is an indicator of new colonization, population fluctuation, natural selection, extinction, and recolonization on a local scale (Grant and Bowen 1998). In addition, RFLPs of mtDNA provide evidence of female-mediated gene flow, founder events, and other population-level processes (Birky et al. 1983; Ferris and Berg 1987).

This study examines the population structure of *Solea senegalensis* using RFLP analysis of the 16S rRNA, 12S rRNA, and cytochrome *b* mtDNA genes. These results represent the first attempt to clarify the genetic relationships among populations of this species along the southwestern Iberian peninsula and to assess the population structure of this species using RFLPs of mtDNA.

TABLE 1.—Fragment size estimates in base pairs (bp) of all patterns generated by the endonucleases in the mtDNA segments for analysis of *Solea semegalensis* populations.

mtDNA segment (bp)	Restriction endonuclease	Restriction fragments and sizes (bp)	Total fragment size (bp)
12S rRNA (430)	*Mae* I	A: 200 + 170	370
		C: 230 + 200	430
16S rRNA (620)	*Hae* III	A: 350 + 250	600
		B: 620	620
	Vsp I	B: 620	620
	Bln I	A: 500 + 120	620
		B: 620	620
		C: 400 + 120 + 100	620
	Mbo II	A: 300 + 150 + 100	550
		B: 620	620
		C: 400 + 150 + 100	650
Cytochrome *b* (340)	*Mae* II	A: 200 + 150	350
		C: 100	100
	Mae III	A: 190 + 100	290
		C: 100	100

Methods

A total of 109 adult *S. senegalensis* (average weight, 200 g) were collected during 2001–2002 from commercial fisheries at five Spanish and Portuguese locations (Figure 1). Approximately 25 mg of muscle tissue was collected and preserved at −80°C until the DNA extraction was conducted by means of the DNA Fastkit before using the Fastprep equipment (Qbiogene). Polymerase chain reaction (PCR) amplifications of the 12S rRNA, 16S rRNA, and cytochrome *b* mitochondrial gene segments were performed using the respective sets of primers 12SAL–12SBH, 16SARL–16SBRH, and L14841–H15149 (Kocher et al. 1989; Palumbi and Wilson 1990; Cunningham et al. 1992). The DNA amplifications were performed in 50-μL volumes containing 2 units of *Taq* polymerase, 5 μL of 10× reaction buffer, 0.2–0.5 mM deoxynucleotide triphosphate mix, 1.5 mM $MgCl_2$, approximately 100–150 ng of DNA, and 0.5 μM of each primer. The PCR amplification conditions were as follows: one preliminary denaturation step at 94°C for 5 min followed by 35 cycles in which denaturation was carried out at 94°C for 1 min followed by annealing for 1 min at 52°C for *16S* rRNA, 57°C for 12S rRNA, and 50°C for cytochrome *b*, and primer extension at 72°C for 1.5 min. The PCR cycles were followed by a final extension at 72°C for 5 min.

Two PCR products from each gene segment were sequenced and verified using mtDNA sequences from GenBank. WEBCUTTER 2.0 software (http://www.firstmarket.com/cutter/cut2.html) was used to identify restriction sites in these sequences. We selected endonucleases with one or more restriction sites for RFLP analyses. Amplicons of the 16S rRNA gene from each individual were digested with the restriction enzymes *Bln* I (C↓CTAGG), *Hae* III (GG↓CC), *Vsp* I AT↓TAAT, and *Mbo* II (GAAGA [N_8]). The enzyme *Mae* I (C↓TAG) was used for the 12S rRNA segment and the enzymes *Mae* II (A↓CGT) and *Mae* III (↓GTNAC) for cytochrome *b*. The digestion temperature for all enzymes was 37°C, which was maintained for 2 h; reactions were then subjected to 65°C for 10 min to stop the restriction activity.

Digested segments were then separated electrophoretically on a 2% agarose gel in tris–borate–EDTA buffer, stained with ethidium bromide, and viewed under ultraviolet light. The DNA fragment sizes were compared with the PCR marker XIV (Promega) run on the same gel.

Statistical analysis.—Haplotypic diversity (*h*) and haplotype frequencies were estimated by the number of haplotypes of mtDNA segments obtained using the GENETIX 4.01 program (Belkir et al. 1996). Genetic differentiation, given by the overall genetic differentiation index (F_{ST}) and pairwise comparisons among populations were quantified with the theta of Weir and Cockerham (1984) using GENETIX 4.01 (Weir and Cockerham 1984; Belkir et al. 1996). The significance of this parameter was evaluated through random permutation procedures (minimum of 10,000 permutations). In addition, Bonferroni adjustment was performed (Rice 1989). A genetic distance matrix based on Nei genetic distance (Nei 1972) was constructed using the NEIGHBOUR.EXE program in the PHYLIP 3.5 package (Felsenstein 1995). A consensus unrooted neighbor-joining tree and bootstrap percentages were generated by means of the PHYLIP software.

Results

The RFLP analyses revealed that the most variable segment was that for 16S rRNA (Table 1). All seven endonucleases detected at least one restriction site, and

TABLE 2.—Composite haplotypes, haplotype frequencies by population, haplotypic diversity (*h*) and number of *Solea senegalensis* individuals per population (*N*).

Composite haplotypes and statistic	16S rRNA				12S rRNA (*Mae* I)	Cytochrome *b*		Population				
	Hae III	*Vsp* I	*Bln* I	*Mbo* II		*Mae* II	*Mae* III	Lisbon	Portimao	Punta Umbria	Valdela-grana	Rio San Pedro
1	A	B	A	A	A	A	A	0.20	1.00	0.60	0.68	0.80
2	A	B	A	A	A	C	A					0.10
3	A	B	B	A	A	A	A			0.05	0.10	0.10
4	A	B	A	A	C	A	A				0.06	
5	A	B	B	C	A	A	A				0.06	
6	B	B	B	A	A	A	A				0.03	
7	A	B	C	A	A	A	A				0.06	
8	A	B	A	B	A	A	A	0.05		0.05		
9	A	B	A	C	A	A	A	0.05				
10	A	B	A	A	A	A	C	0.30				
11	A	B	A	A	A	A	A	0.20				
12	A	B	B	A	A	C	C	0.20				
13	B	B	A	A	A	A	A			0.25		
14	A	B	B	B	A	A	A			0.05		
h								0.183	0.00	0.088	0.107	0.088
N								20	20	29	20	20

six (*Hae* II, *Bln* I, *Mbo* II, *Mae* I, *Mae* II, *and Mae* III) produced polymorphic restriction fragments. Only *Vsp* I was not informative for the mtDNA regions analyzed.

A total of 14 composite haplotypes were found for the analyzed gene regions where the values of *h* did not exceed 0.18 (18%) in any of the five analyzed populations (Table 2). Composite haplotype frequencies per population identified three haplotypes that occurred in more than one sample. Haplotype 1 occurred in four or more individuals in all samples. Haplotype 3 was found in one or two individuals among three geographically proximate locations in the Gulf of Cadiz. Single individuals with haplotype 8 were found in the Punta Umbria and Lisbon collections. The 11 remaining haplotypes were unique within the populations.

Pairwise estimates of F_{ST} values ranged between 0.000 and 0.293 (Table 3). Significant differences were found in 4 of the 15 pairwise comparisons, all involving the Lisbon samples versus those of the other locations. Although no other significant comparisons were observed, the lowest F_{ST} occurred among samples from the Gulf of Cadiz. Thus, two distinct genetic groups were identified (Lisbon and all others) and there may be three (Lisbon, Portimao, and the Gulf of Cadiz). The topology and bootstrap values (54–88) of Figure 2 support the existence of these three groups, as does the high level of structure for the Lisbon population.

TABLE 3.—Fragment sizes of all patterns of the mtDNA segments and representation of how the haplopypes appeared in agarose gels.

Fragment size (bp)	16S rRNA									12S rRNA (*Mae* I)		Cytochrome *b*			
	Hae III		*Vsp* I	*Bln* I			*Mbo* II					*Mae* II		*Mae* III	
	A	B	(B)	A	B	C	A	B	C	A	C	A	C	A	C
620		—	—		—			—							
500				—		—									
450															
400									—						
350	—														
300							—								
250	—														
240											—	—			
200										—	—				
190															—
170										—		—			
150															
120															
100												—	—	—	—

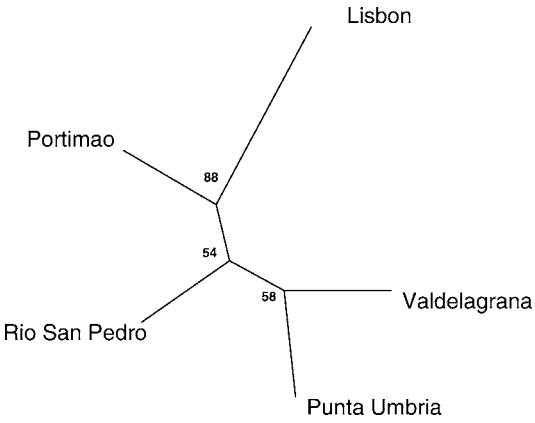

FIGURE 2.—Consensus neighbor-joining tree showing the bootstrap values of the five surveyed *Solea senegalensis* populations based on a Nei (1972) genetic distance matrix.

Discussion

Detecting genetic variation in mtDNA between organisms depends mainly on the evolutionary rate of genes and the number of nucleotide bases surveyed (Moritz et al. 1987; Papasotiropoulos et al. 2002). We found that the largest gene region (16S rRNA) showed the highest haplotype diversity. In other species, for which the analyzed segment of 16S rRNA is almost the same size as that for other genes (such as cytochrome *b*), no significant differences in variation were observed. Other mitochondrial genes such as cytochrome *b* are intraspecifically conserved but contain enough interspecific heterogeneity to produce species-specific patterns (Carr and Marshall 1991). For example, several differences were found among *Solea solea*, plaice *Pleuronectes platessa*, European flounder *Platichthys flesus*, and Greenland halibut *Reindhartius hippoglossoides* through the use of PCR–RFLP analysis of cytochrome *b* (Céspedes et al. 1999). In addition, several differences have been reported among five pleuronectiform species that inhabit the Adriatic and Mediterranean basins (Tinti et al. 1999, 2000) by researchers using the nucleotide variation of cytochrome *b* and control region segments. Other segments, such as those for 16S rRNA and 12S rRNA, are also considered useful for the establishment of phylogenetic relationships among pleuronectiform species (Berendzen and Wheaton 2002; Infante et al. 2004). Nevertheless, intrapopulation variation studies using mtDNA segments have not been reported for *Solea senegalensis* or other congeneric species.

Although neither cytochrome *b* nor 12S rRNA were highly informative for population structure analysis in *S. senegalensis* using PCR–RFLP, their added variation was important for generating composite haplotypes unique to some individuals and populations and to obtain values for *h*. Analogous to genetic diversity, haplotypic diversity is a good estimator of genetic health, levels of exploitation, and population size (Avise 2004). Overall, our *h* values are fairly low in comparison with other values obtained for pelagic species using mtDNA RFLP (e.g., Grant and Bowen 1998; Klossa-Kilia et al. 2002; Karaiskou et al. 2003). These values corroborate the low levels of genetic diversity obtained for *Solea senegalensis* and *Solea solea* when isoenzymes were used to study the genetic structure of Portuguese populations (Cabral et al. 2003). However, the higher values of *h* found at Lisbon and Valdelagrana are consistent with more females partaking in the reproduction process in these areas and probably points to the existence of some degree of spawning philopatry, since this behaviour have been reported in other flatfish species (Stepien 1999).

The absence of apparent genetic structure among Gulf of Cadiz populations based on RFLP analysis of mtDNA segments may reflect high gene flow and homogenization within this area. Larval transport via the Atlantic Superficial Current would mediate such gene flow in the Gulf of Cadiz (Michina and Rebordinos 1997). The north-to-south pattern of this current and the proximity of *Solea senegalensis* populations would promote the admixture of these populations. Some unique haplotypes in locations like Punta Umbria, Rio San Pedro, and Valdelagrana may reflect some differentiation among these populations, but the presence of these haplotypes is not extensive enough for them to be considered subpopulations of the species.

On the other hand, the genetic differentiation observed in Lisbon is very likely a consequence of the coastal topography of Setubal Bay. Previous population studies along the Portuguese coast related the observed genetic differentiation to canyons in this area that acted as barriers to gene flow (Cabral et al. 2000, 2003). Such level of structure is corroborated by elevated and significant pairwise values of F_{ST} (Table 4). Further isolation suggested by fixation of haplotype 1 in the Portimao population (located on the northern boundary of the Gulf of Cadiz) and the absence of unique southern Gulf haplotypes (types 2 to 7) in Portuguese populations (Lisbon and Portimao; Table 2) provides evidence for gene flow with a north-to-south pattern. Separation between Portuguese and Gulf populations is also suggested by the topology of the neighbor-joining tree. A possible explanation, since we are using mtDNA markers, is that females are less likely than males to migrate long distances, instead staying in larger reproductive areas like Lisbon.

TABLE 4.—Pairwise values of F_{ST} among populations of *S. senegalensis*. Asterisks denote values that were significant at the 0.05 level after sequential Bonferroni adjustment.

Population F_{ST}	Lisbon	Portimao	Punta Umbria	Valdelagrana	Rio San Pedro
Lisbon		0.293*	0.269*	0.277*	0.244*
Portimao			0.036	0.066	0.036
Punta Umbria				0.016	−0.019
Valdelagrana					0.021
Rio San Pedro					

Marine flatfishes are characterized by weak or no population differentiation on a large spatial scale, especially in species that display long larval development (Bouza et al. 1997; Waples 1998; Hourau et al. 2002). However, even in those species contradictory results have been obtained. For instance, *Solea solea* have shown a lack of genetic differentiation within the Mediterranean Sea populations (Koutolas et al. 1995), while strong genetic differentiation has been found between those populations and northern European populations (Exadactylos 1998). Another example is the Japanese flounder *Paralichthys olivaceus*, for which low genetic differentiation has been reported by researchers using microsatellite loci (Sekino and Hara 2001) while mtDNA sequencing analysis has revealed significant genetic heterogeneity between two adjacent populations in the same area (Fujii and Nishida 1997).

In the case of *Solea senegalensis*, the observed genetic differentiation in the Lisbon population despite the marine habits of the species is consistent with homing behavior in. In addition, the short larval development of the species (19 d) and the enclosed shape of Setubal Bay in Lisbon promote larval retention. In light of the natural history of *Solea senegalensis*, more populations with defined population structure are expected; however, the use of finer scale markers should be considered in order to detect such levels of heterogeneity (Diaz-Ferguson 2004).

In conclusion, the significant values of F_{ST} observed in Lisbon and the existence of unique haplotypes in almost all surveyed populations are consistent with some degree of structure among the analyzed populations. Although the pairwise F_{ST} values support the existence of only two groups (Lisbon and the other populations), conservative management may recognize three groups (Lisbon, Portimao, and the other populations) based on the topology of the neighbor-joining consensus tree. These preliminary observations are intended to guide management and promote further studies to clarify the geographic and temporal details of this apparent structure. Such studies should include further sampling at both previously studied and other locations over a spatial scale covering the whole distributional range of the species. Furthermore, a comparative analysis involving both mtDNA and finer scale nuclear markers (microsatellites) will be necessary to corroborate the observed structure and establish proper management and conservation programs for *Solea senegalensis* along the southern Atlantic coast.

Acknowledgments

This research was supported in part by an INTERREG III A project (Ordenamiento Pesquero Andalucia Marruecos) grant from the Agencia Espanola de Cooperacion Internacional and by a grant from the Spanish Junta de Andalucia (CV1219). We thank Michael Canino for his comments and help writing the paper in English.

References

Avise, J. 2004. Molecular markers, natural history, and evolution. Sinauer Associates, Sunderland, Massachusetts.

Belkir, K., P. Borsa, J. Goudet, L. Chkhi, and F. Bonhomme. 1996. Genetix: logiciel sous Windows pour la génétíque des populations. [Genetix: Windows software for population genetics.] Laboratoire Génome et Populations, Université de Montpellier II, Montpellier, France.

Berendzen, P., and W. Wheaton. 2002. Phylogenetic relationships of Pleuronectiformes based on molecular evidence. Copeia 2002:642–652.

Birky, W., T. Maruyama, and P. Fuerst. 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplast, and some results. Genetics 103:513–527.

Bouza, C., L. Sanchez, and P. Martinez. 1997. Gene diversity analysis in natural populations and cultured stocks of turbot (*Scophthalmus maximus* L.). Animal Genetics 28:28–36.

Cabral, H. 2000. Distribution and abundance patterns of flatfishes in the Sado Estuary, Portugal. Estuaries 23:351–358.

Cabral, H., F. Castro, D. Linhares, and D. Alexandrino. 2003. Genetic differentiation of *Solea solea* (Linnaeus, 1758) and *Solea senegalensis* (Kaup, 1858) (Pisces: Pleuronectiformes) from several estuarine systems of the Portuguese coast. Scientia Marina 67(1):43–52.

Carr, S. M., and H. D. Marshall. 1991. A direct approach to the measurement of genetic variation in fish populations: applications of the polymerase chain reaction to studies

of Atlantic cod, *Gadus morhua* L. Journal of Fish Biology 39:101–107.

Céspedes, A., T. García, E. Carrera, I. González, P. Hernández, and R. Martín. 1999. Identification of sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR amplification of the 5S rDNA gene. Journal of Agricultural and Food Chemistry 47:1046–1050.

Cunningham, C. W., N. W. Blackstone, and L. W. Buss. 1992. Evolution of king crabs from hermit crab ancestors. Nature (London) 355:539–542.

Diaz-Ferguson, E. 2004. [Genetic characterization of natural populations of *Solea senegalensis* in the Gulf of Cadiz.] Doctoral dissertation. University of Cadiz, Cadiz, Spain.

Dinis, M. T., L. Ribeiro, F. Soares, and C. A. Sarasquete. 1999. Review of the cultivation potential of *Solea senegalensis* in Spain and Portugal. Aquaculture 176:27–38.

Exadactylos, A., A. Geffen, and J. Thorpe. 1998. Population structure of the Dover sole, *Solea solea* L., in a background of high gene flow. Journal of Sea Research 40:117–129.

Felsenstein, J. 1995. Phylip (Phylogeny Inference Package). University of Washington, Seattle.

Ferál, J. 2002. How useful are the genetic markers in attempts to understand and manage marine biodiversity? Journal of Experimental Marine Biology and Ecology 246:121–145.

Ferris, S., and W. Berg. 1987. The utility of mitochondrial DNA in fish genetics and fishery management. Pages 277–299 *in* N. Ryman and F. Utter, editors. Population genetics and fishery management. University of Washington, Seattle.

Fujii, F., and M. Nishida. 1997. High sequence variability in the mitochondrial DNA control region of the Japanese flounder *Paralichthys olivaceus*. Fisheries Science 63:906–910.

Grant, W. S., and B. W. Bowen. 1998. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. Journal of Heredity 89:415–426.

Hourau, G., D. Rijnsdorp, W. Van der Veer, T. Stam, and L. Olsen. 2002. Population structure of plaice (*Pleuronectes platessa* L.) in northern Europe: microsatellites revealed large-scale spatial and temporal homogeneity. Molecular Ecology 11:1165–1176.

Imsland, A. K., A. Foss, L. E. C. Conceiçao, M. T. Dinis, D. Delvare, E. Schram, A. Kamstra, P. Rema, and P. White. 2003. A review of the culture potential of *Solea solea* and *Solea senegalensis*. Reviews in Fish Biology and Fisheries 13:379–407.

Infante, C., G. Catanese, and M. Manchado. 2004. Phylogenetic relationships among ten sole species (Soleidae, Pleuronectiformes) from the Gulf of Cadiz (Spain) based on mitochondrial DNA sequences. Marine Biotechnology 6:621–624.

Karaiskou, N., A. P. Apostolidis, A. Triantafyllidis, A. Kouvatsi, and C. Triantaphyllidis. 2003. Genetic identification and phylogeny of three species of the genus *Trachurus* based on mitochondrial DNA analysis. Marine Biotechnology 5:493–504.

Klossa-Kilia, E., M. Prassa, V. Papasitoropoulos, S. Alahiotis, and G. Kilias. 2002. Mitochondrial DNA diversity in *Atherina boyeri* populations as determined by RFLP analysis of three mtDNA segments. Heredity 89:363–370.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo, and F. X. Villablanca. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences of the USA 86:6196–6200.

Koutolas, G., F. Bonhomme, and P. Borsa. 1995. Genetic structure of the common sole, *Solea vulgaris*, at different geographic scales. Marine Biology 122:361–375.

Lande, R. 1988. Genetics and demography in biological conservation. Science 241:1455–1460.

Michina, S. R., and L. Rebordinos. 1997. Genetic differentiation in marine and estuarine natural populations of *Crassostrea angulata*. Marine Ecology Progress Series 154:167–174.

Moritz, C. 1994. Applications of mitochondrial DNA analysis to conservation: a critical review. Molecular Ecology 3:401–411.

Moritz, C., T. E. Dowling, and W. M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annual Review of Ecology and Systematics 18:269.

Mustafa, S. 1999. Genetics in sustainable fisheries management. Fishing New Books, London.

Nei, M. 1972. Genetic distance between populations. American Naturalist 106:283–292.

Ovenden, J. R. 1990. Mitochondrial DNA and marine stock assessment: a review. Australian Journal of Marine and Freshwater Research 41:835–853.

Palumbi, S. R., and A. C. Wilson. 1990. Mitochondrial DNA diversity in the sea urchins. Evolution 44:403–415.

Papasotiropoulos, V., E. Klossa-Kilias, G. Kilias, and S. Alahiotis. 2002. Genetic divergence and phylogenetic relationships in grey mullets (Teleostei: Mugilidae) based on PCR–RFLP analysis of mtDNA segments. Biochemical Genetics 40:71–86.

Pinheiro, A., C. Teixeira, A. Rego, J. Marques, and H. Cabral. 2005. Genetic and morphological variation of *Solea lascaris* (Risso, 1810) along the Portuguese coast. Fisheries Research 73:67–78.

Rice, W. 1989. Analysing tables of statistical tests. Evolution 43:223–225.

Rodriguez, A., and R. B. Rodriguez. 1980. First record of *Solea senegalensis* Kaup 1858 (Heterosomata, Soleidae) from the Mediterranean Sea. Investigaciones Pesqueras 44:291–295.

Rodriguez Martinez, R. 1984. Biología y cultivo de *Solea senegalensis* Kaup, 1858 en el Golfo de Cádiz. [Biology and culture of *Solea senegalensis* Kaup, 1858 in the Gulf of Cadiz.] Doctoral dissertation. University of Seville, Seville, Spain.

Sekino, M., and M. Hara. 2001. Application of microsatellite markers to population genetics studies of the Japanese flounder *Paralichthys olivaceus*. Marine Biotechnology 3:572–579.

Stepien, C. 1999. Phylogeographical structure of the Dover sole *Microstomus pacificus*: the larval retention hypothesis and genetic divergence along the deep continental

slope of the northeastern Pacific Ocean. Molecular Ecology 8:923–939.

Tinti, F., A. Colombari, M. Vallisneri, C. Piccinetti, and A. Stagni. 1999. Comparative analysis of a mitochondrial DNA control region fragment amplified from three Adriatic flatfish species and molecular phylogenetics of Pleuronectiformes. Marine Biotechnology 1:20–24.

Tinti, F., C. Piccinetti, S. Tommasini, and M. Vallisneri. 2000. Mitochondrial DNA variation, phylogenetic relationships, and evolution of four Mediterranean genera of soles (Soleidae, Pleuronectiformes). Marine Biotechnology 2:274–284.

Waples, R. S. 1998. Separating the wheat from the chaff: patterns of genetic differentiation in high-gene-flow Species. Journal of Heredity 89:438–450.

Weir, B. S., and C. C. Cockerham. 1984. Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370.