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Phylogenetic Relationships Among Ten Sole Species (Soleidae, Pleuronectiformes) from the Gulf of Cádiz (Spain) Based on Mitochondrial DNA Sequences

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Abstract: The entire sequence of the mitochondrial cytochrome *b* gene and 2 partial sequences of the ribosomal RNA12S and 16S genes have been used to study the molecular phylogeny in 10 species of soles belonging to the genera *Solea*, *Monochirus*, *Microchirus*, *Dicologlossa*, and *Synaptura* from the Atlantic waters of the Gulf of Cádiz (Spain). The results obtained by means of different phylogenetic analyses (maximum likelihood, maximum parsimony, and neighbor-joining) were quite similar, supporting the monophyly of the *Solea* species. Nevertheless, they favor the differentiation of *Dicologlossa cuneata* and *Dicologlossa hexophthalma* in 2 distinct genera, since the most closely related species to the last one is *Microchirus azevia*. The fact that *M. azevia* is also more closely linked to *Monochirus hispidus* than to its congeneric *Microchirus boscanion* argues in favor of a taxonomic reorganization of these genera.

Key words: Soleidae, Pleuronectiformes, phylogeny, *Solea*, *Dicologlossa*, cytochrome *b*.

INTRODUCTION

Soleidae are benthic flatfishes that share several morphologic characteristics. They have both eyes on the right side of the body, with the body oval in outline and strongly compressed, and the preoperculum covered by skin and scales (Quéro et al., 1986; Bauchot, 1987). They preferentially inhabit sandy or sand-mud bottoms of the continental shelf and slope, from close to shore down to 1300 m, feeding mainly on a wide range of small bottom-living organisms like crustaceans, mollusks, and marine worms (Quéro et al., 1986). Seven genera with 17 different species are distributed in the northeastern Atlantic and the Mediterranean (Quéro et al., 1986). In the Atlantic waters of the Gulf of Cádiz, the area where this survey is focused, Soleidae are represented by 6 different genera including 14 species: *Buglossidium* (*B. luteum*), *Solea* (*S. vulgaris*, *S. senegalensis*, *S. lascaris*, *S. kleinii*, and *S. impar*), *Microchirus* (*M. azevia*, *M. boscanion*, *M. ocellatus*, and *M. variegatus*), *Monochirus* (*M. hispidus*), *Synaptura* (*S. lusitanica*), and *Dicologlossa* (*D. cuneata* and *D. hexophthalma*) (Quéro et al., 1986; Bauchot, 1987). Most of them have high commercial value in Spain, including common sole, Klein's sole, sand sole, Senegalese sole, wedge sole, bastard sole, or the six-eyed sole.

Traditional systematic studies of the Soleidae have been based on morphologic features. In this sense the re-

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sults of several authors showed great differences in the number and nomenclature of taxa depending on the relevance assigned to the characters used (Bini, 1968; Torchio, 1973; Tortonese, 1975; Quéro et al., 1986; Bauchot, 1987; Ben-Tuvia, 1990). Within the genus *Solea* there was a classic subdivision into 2 subgroups based on the shape of the anterior nostril on the blind side: *Pegusa*-like (nostril enlarged) and *Solea*-like (nostril not enlarged). The first one included 4 species: *S. kleinii*, characterized by a cupola-shaped nostril, and *S. nasuta*, *S. lascaris*, and *S. impar*, with a rossete-shaped nostril. In contrast, the species *S. vulgaris*, *S. aegyptiaca*, and *S. senegalensis* were included in the *Solea*-like subgroup on the basis of a normal-shaped nostril (Quéro et al., 1986; Bauchot, 1987). Nevertheless, further reappraisals based on morphologic data (Ben-Tuvia, 1990) and mitochondrial DNA (mtDNA) partial sequences of the cytochrome *b* (*cytb*) and ribosomal RNA 16S genes (Tinti and Piccinetti, 2000) supported a taxonomy in which only 4 species are maintained: *S. vulgaris*, *S. senegalensis*, *S. kleinii*, and *S. lascaris*, each branching off independently from a common ancestor. However, some authors argued later in favor of maintaining *S. aegyptiaca* and *S. impar* as valid species according to morphologic and phylogenetic data (Borsa and Quignard, 2001). So, this issue remains controversial.

In the case of the genus *Microchirus*, the 2 species analyzed show enough morphologic differences to be easily distinguished. The species *M. boscanion* shows small dark cross-bands on the body, ending in conspicuous dark patches on dorsal and anal fins. On the contrary, in *M. azevia* these bands are absent, though ocassionally juveniles can present some large indistinct spots (Quéro et al., 1986; Bauchot, 1987). The genus *Monochirus*, only represented by the species *M. hispidus*, is characterized by the lack of a pectoral fin on the blind side, and by the presence of a nostril on the eyed side that is tubular shaped and very long, usually reaching to the pupil of the lower eye (Quéro et al., 1986; Bauchot, 1987). The genus *Synaptura* is also represented by only one species, *S. lusitanica*, characterized by the presence of dorsal and anal finrays confluent with the caudal fin (Quéro et al., 1986; Bauchot, 1987).

The genus *Dicologlossa* groups 2 very different species in appearance, *D. hexophthalma* and *D. cuneata*. The former presents on the eyed side a series of characteristic conspicuous black spots ringed by a narrow light border (named ocelli), 3 along the dorsal fin and 3 along the anal fin (Quéro et al., 1986; Bauchot, 1987). The second of the species, *D. cuneata*, does not show any kind of ocelli, but has a body elongate and the supratemporal branch forming an angular S-shape characteristic of the genus (Quéro et al., 1986; Bauchot, 1987).

Several morphologic studies have focused on defining the phylogenetic relationships of flatfishes (Chapleau, 1993; Hensley, 1997; Cooper and Chapleau, 1998a, 1998b; Hoshino and Amaoka, 1998; Hoshino, 2001). These surveys have been complemented with others based on enzyme polymorphisms (Verneau et al., 1994; Kotoulas et al., 1995; Borsa et al., 1997; Exadactylos and Thorpe, 2001). Nevertheless, it seems clear that the development of molecular techniques based on DNA could help to elucidate some controversial aspects of flatfish systematics. So, analyses of mitochondrial markers, including slowly evolving rRNA genes (Berendzen and Dimmick, 2002) and the highly variable mtDNA control region (Tinti et al., 1999), have been applied to the establishment of phylogenetic relations between flatfishes belonging to different families. More specifically, partial sequences of the *cytb* and ribosomal RNA 16S mitochondrial genes have proven to be useful in the analysis of the systematics between several species of Mediterranean soles (Tinti et al., 2000) and between Atlanto-Mediterranean *Solea species* (Tinti and Piccinetti, 2000).

The aim of this work was to assess the phylogenetic relationships among 10 species belonging to 5 different genera of the family Soleidae. All these species inhabit the Atlantic waters of the Gulf of Cádiz (Spain), and some of them have high commercial value. In each case we have obtained the entire sequence of *cytb* gene, and a partial sequence of the 16S and 12S rRNA genes. The results of the molecular analyses have been evaluated in relation to others previously published.

Materials and Methods

All species included in the present study (Table 1) were collected during monthly samplings of demersal fishes carried out as a part of the scientific project "Fisheries Resources of the Gulf of Cádiz", supported by the "Consejería de Agricultura y Pesca" of the "Junta de Andalucía" (Spain). Soles were classified according to Bauchot (1987). A muscular portion of each of the specimens was excised and kept at −80°C. Total genomic DNA was isolated from 150 mg of the tissue using FastDNA kit for 40 seconds and speed setting 5 in the Fastprep FG120 instrument (Bio101, Inc.). All DNA isolation procedures were performed fol-

Table 1. Species of Soleidae Included in the Analysis

Species	Author, Year	Common name
Solea lascaris	Risso, 1810	Sand sole
Solea senegalensis	Kaup, 1858	Senegalese sole
Solea vulgaris	Quensel, 1806	Common sole
Solea kleinii	Risso, 1827	Klein's sole
Microchirus azevia	Capello, 1867	Bastard sole
Microchirus boscanion	Chabanaud, 1926	Lusitanian sole
Monochirus hispidus	Rafinesque, 1814	Whiskered sole
Dicologlossa cuneata	Moreau, 1881	Wedge sole
Dicologlossa hexophthalma	Bennett, 1831	Six-eyed sole
Synaptura lusitanica	Capello, 1868	Portuguese sole

lowing the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out in a 25-μl reaction volume containing 16.75 μl sterilized distilled water, 2.5 μl deoxynucleoside triphosphate (dNTPs 10 mM), 2.5 μl of 10× buffer, 1 μl $MgCl_2$ (50 mM), 0.5 μl each primer (10 μM), and 0.25 μl Bio*Taq* DNA polymerase (Bioline). All primers were designed using the software Oligo Version 6.82 (Medprobe). Fragments of the rRNA 12S gene were amplified using the forward primer 12S●1 (5′-GAC AGCTACGACACAAACTGCGATTAGATACC-3′) and the reverse primer 12S●2 (5′-TGCACCTTCCAGTACACTTACCATGTTACGAC-3′). For the rRNA 16S gene, the primers used were 16S●1 (5′-CCTCGCCTGTTTACCAAAAACATCGCCTC-3′) as forward, and 16S●2 (5′-TAATAGCGGCTGCACCATTAGGATGTCCTG-3′) as reverse. The thermal cycle profile for rRNA genes was 30 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. For the *cytb* gene the primers used for each of the species are given in Table 2. In the cases of *S. lascaris*, *S. senegalensis*, and *S. kleinii*, it was necessary to amplify 2 overlapping products to obtain the complete sequence of the gene. PCR conditions were similar as those used for rRNA genes, differing only in the annealing temperature (see Table 2) and the extension time of 90 seconds instead of 1 minute. PCR products were electrophoresed on a 2% agarose gel and visualized via ultraviolet transilumination before sequencing.

Double-stranded DNA products were purified using a PCR product purification kit (Marlingen Bioscience) and subsequently used for direct cycle sequencing with BigDye Terminator Version 3.1 kit (Applied Biosystems). All sequencing reactions were performed according to the manufacturer's instructions on a 377 DNA sequencer (Applied Biosystems). Primers used were the same as those for PCR.

Nucleotide sequences were analyzed using the computer programs Sequencing Analysis Version 3.4.1 (Applied Biosystems) and Seqman Version 5.51 (DNASTAR), and further aligned with the Megalign 5.51 package (DNASTAR). The sequences obtained have been deposited in GenBank/EMBL/DDBJ with the following accession numbers: AB125234 to AB125244 (rRNA 12S), AB125245 to AB125255 (rRNA 16S), AB125325 to AB125335 (*cytb*). Amino acid *cytb* sequences were translated from nucleotide sequences applying vertebrate mitochondrial DNA genetic code.

The base compositional bias (Irwin et al., 1991) for each species was calculated for the 3 mitochondrial fragments, and for each codon position of *cytb* sequences. In order to assess if transitions reached saturation in the compared species, the number of substitutions was plotted against uncorrected genetic *p*-distance for each pairwise ingroup comparison.

The Modeltest Version 3.06 software (Posada and Crandall, 1998) was employed as a guide to determine the best-fit maximum likelihood (ML) model as described by Cunningham et al. (1998). Additionally, ML, maximum parsimony (MP), and neighbor-joining (NJ) (Saitou and Nei, 1987) analyses were carried out both on individual and on combined data sets using PAUP*4.0b10 (Swofford, 2000). The degree of confidence assigned to nodes in trees was determined by bootstrapping (Felsenstein, 1985) with 2000 replicates (Hedges, 1992). The MP analyses were performed applying the heuristic search option with tree bisection-reconnection (TBR) branch-swapping and 1000 random-taxon-addition replicates. Heuristic MP bootstrap

Table 2. Primers Used to Obtain the Entire Sequence of the *cytb* gene for each species

Species	Forward/reverse	Annealing temp. (°C)
Solea lascaris	Glu●1/cytb●2, cytb●1/12S●4 Glu●1/cytb.2	50, 60
Solea senegalensis	Glu●1/cytb●2, cytb●1/12S●6 Glu●1/cytb.2	60
Solea vulgaris	Glu●1/Thr●2 Glu●1/cytb.2	60
Solea kleinii	Glu●1/cytb●2, cytb●3/12S●4 Glu●1/cytb.2	50, 58
Microchirus azevia	Glu●1/Thr●2 Glu●1/cytb.2	56
Microchirus boscanion	Glu●1/Thr●2 Glu●1/cytb.2	50
Monochirus hispidus	Glu●1/Pro●2 Glu●1/cytb.2	50
Dicologlossa cuneata	Glu●1/Pro●2 Glu●1/cytb.2	50
Dicologlossa hexophthalma	Glu●1/Thr●2 Glu●1/cytb.2	56
Synaptura lusitanica	Glu●1/Pro●2 Glu●1/cytb.2	54
Platichthys flesus	Glu●1/Thr●2 Glu●1/cytb.2	60
Glu●1	5′-GGGGATTTTAACCTCAGGCGTTCAGTTTAC-3′ Glu●1/cytb.2	
Thr●2	5′-GGACTAATCGCTTGAAAAACCACCGTTG-3′ Glu●1/cytb.2	
Pro●2	5′-GCTTTGGGAGTTAGGGGTAGGAGTTGAAATCT-3′ Glu●1/cytb.2	
cytb●1	5′-CTGACCCGATTCTTCACCTTCCACTTCCT-3′ Glu●1/cytb.2	
cytb●2	5′-GGAATTGAGCGGAGGATTGCGTATGC-3′ Glu●1/cytb.2	
cytb●3	5′-GACAACTTCACCCCAGCAAACCCCCTA-3′ Glu●1/cytb.2	
12S●4	5′-TGCACCTTCCAGTACACTTACCATGTTACGAC-3′ Glu●1/cytb.2	
12S●6	5′-TCTCATGTGCTACACCTCGACCTGACGTT-3′ Glu●1/cytb.2	

analyses also consisted of 2000 pseudoreplicates (TBR branch swapping), with 10 random-taxon-addition replicates per pseudoreplicate. In the case of *cytb* amino acid sequences, ML distance matrices were obtained with the program Molphy Version 2.3 (Adachi and Hasegawa, 1996) using the mtREV24 model. Subsequently, Neighbor and Consense programs implemented in Phylip Version 3.6b (Felsenstein, 2004) were employed to build NJ trees and generate the final consensus tree with bootstrap values (1000 replicates) for nodes.

Results

An alignment of 1141 nucleotide sites for *cytb*, 525 for rRNA 12S, and 548 for rRNA 16S was obtained. The number of variable sites ranged from 558 (48.9%) for *cytb* (Figure 1), 201 (38.3%) for rRNA 12S (Figure 2), to 170 (31%) for rRNA 16S (Figure 3). As expected, most of the *cytb* variable sites were found at the third codon position (first, 35.3%; second, 16.3%; third, 95.3%). No nucleotide compositional bias was evident in the rRNA 12S or rRNA 16S gene fragments, while in the *cytb* sequences a bias toward T and C was found at the second and third positions, respectively (Table 3). An anti-G bias was also detected at the second and third positions, a general feature of the mitochondrial genes encoded on the H strand. The compositional bias was high at the second and third codon positions (0.226 and 0.260, respectively), but was considerably lower at the first position (0.040).

The scatter plots of transitions and transversions against genetic distances for each pairwise comparison revealed that transitions become saturated in the rRNA 16S gene when sequence divergence was near 15%, and at the third codon position of *cytb* gene when sequence divergence was about 20% (data not shown). In the *cytb* gene the highest value of the transitions-to-transversions ratio was reached between *M. azevia* and *D. hexophthalma* (3.42), while in the rRNAs fragments, the highest values were found between *S. vulgaris* and *S. senegalensis* (6.5 for rRNA 12S and 8.0 for rRNA 16S). Between distantly related species these values were much lower owing to the double effect of back mutations of transitions and an increasing number of transversions.

After sites of sequence alignment showing missing data and insertions/deletions were removed, rRNA 12S and rRNA 16S variable and phylogenetically informative data were of 123 and 137 pb, respectively. Because of saturation

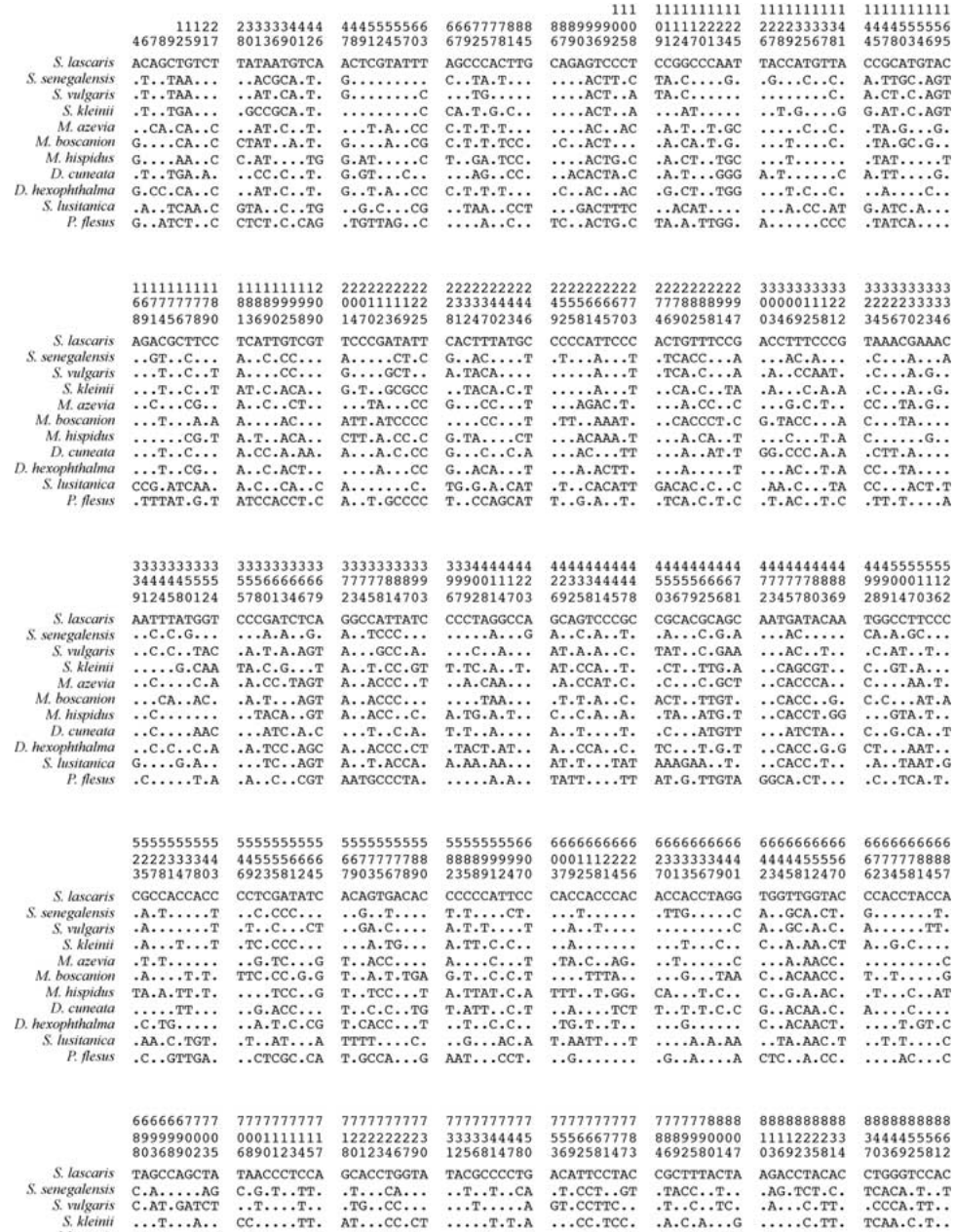

Figure 1. Alignment of *cytb* sequences of the 10 sole species and of the outgroup *Platichthys flesus* (Pleuronectidae). Only variable sites are reported.

of transitions at the third codon position in *cytb* sequences, these sites were also excluded from the phylogenetic analysis. Without considering the nonvariable sites in first and second codon positions in *cytb* sequences, a data set of 454 variable sites was obtained for each of the taxa investigated and used for phylogeny. Tamura-Nei's genetic distances (Tamura and Nei, 1993) based on *cytb*, rRNA 12S, and rRNA 16S sequences are shown in Table 4.

Figure 4 shows the phylogenetic tree constructed on combined (*cytb*, rRNA 16S, and rRNA 12S) sequence data using the ML method. The likelihood ratio test implemented in Modeltest Version 3.06 (Posada and Crandall, 1998) chose the HKY + I + G model of DNA sequence evolution as most appropriate. The model parameters used in ML analysis that resulted in a single tree were as follows: base frequencies were 0.2771, 0.2675, and 0.1888; *Ti*/*tv* = 2.9853; $\alpha = 0.7302$; and the proportion of invariable sites was 0.5456. The same tree topology was identified by MP and NJ methods. In all cases the evolutionary pattern of soles was similar, with 3 distinct lineages. The first one

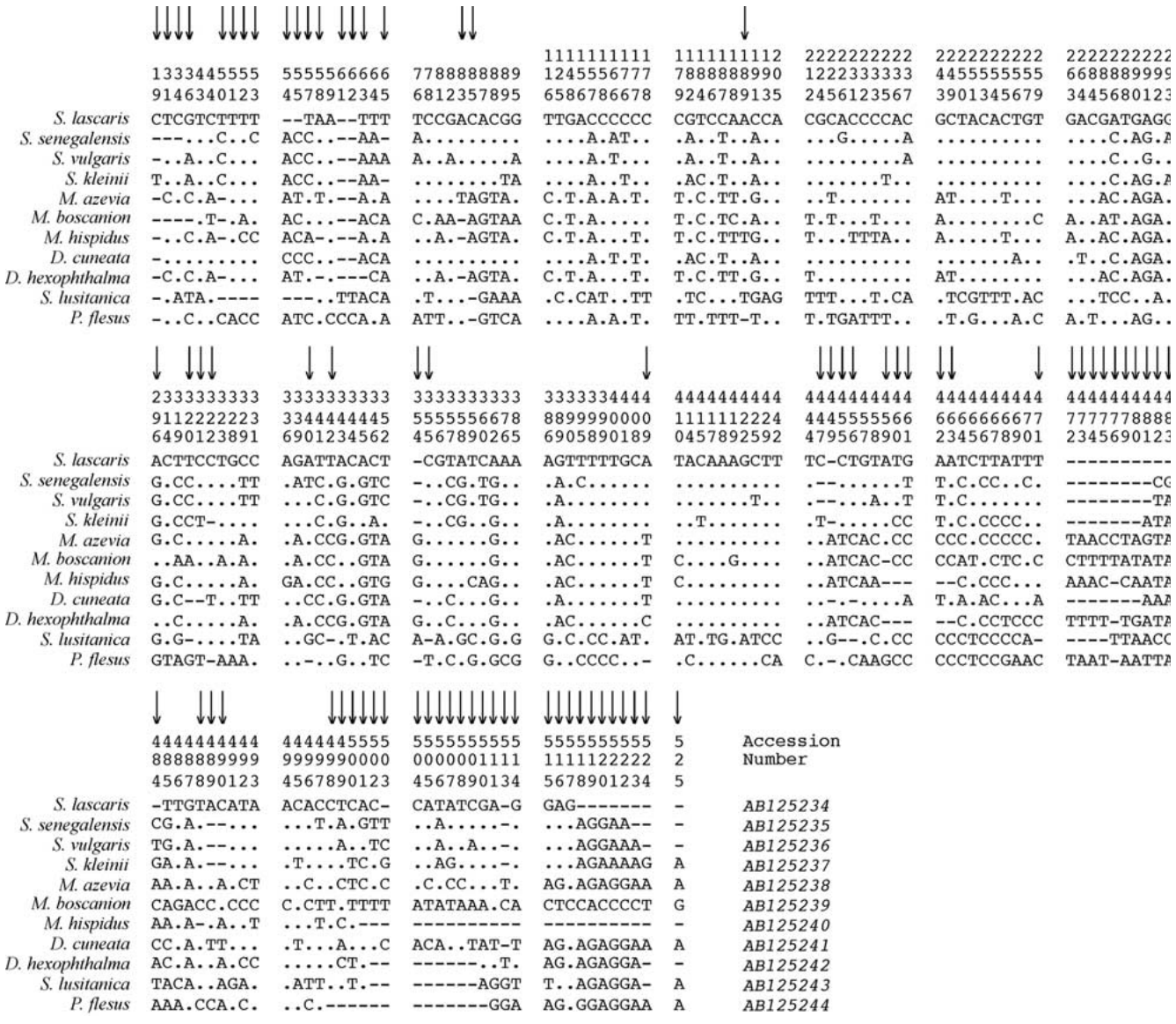

Figure 2. Alignment of rRNA 12S sequences of the 10 sole species and of the outgroup *Platichthys flesus* (Pleuronectidae). Only variable sites are reported. Arrows mark sites carrying insertions/deletions.

included all *Solea* species and *D. cuneata* formed an independent cluster. The *Solea* species appeared as a monophyletic group distinct of *D. cuneata* with all nodes supported with bootstrap values higher than 50%, with the exception of the node showing *S. kleinii* more closely related to *S. senegalensis*/*S. vulgaris* (the most related species) than *S. lascaris*, which was only well supported in the NJ tree. The second lineage grouped *M. hispidus*, *D. hexophthalma*, and the 2 species included in the genera *Microrochirus*. Interestingly, *M. azevia* was more evolutionarily linked to *D. hexophthalma* than to its congeneric *M. boscanion*. These 3 species, together with *M. hispidus*, appeared also as a monophyletic group in relation to the *Solea/D. cuneata* cluster, with bootstrap support for the node always close to 100%. Only the internal node of *M. boscanion/M. hispidus* was supported by less than 50% of bootstrap replicates in ML and MP trees. The last lineage included only one species, *S. lusitanica*, that appeared as the most basal taxon of soles.

The topology of the trees constructed on individual *cytb*, rRNA 16S, and rRNA 12S nucleotide sequences using the 3 different methods also showed on the whole 3 main clusters of taxa, although some slight differences could be noted (Figure 4). For *cytb* gene, the model found to be

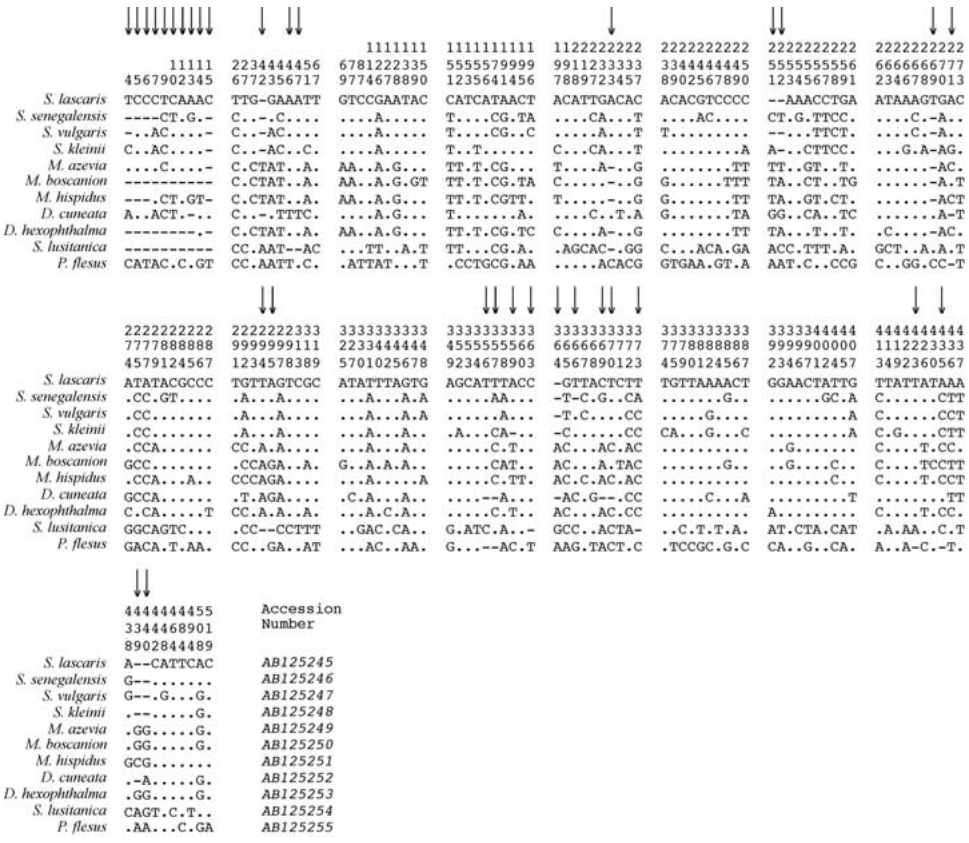

Figure 3. Alignment of rRNA 16S sequences of the 10 sole species and of the outgroup *Platichthys flesus*. Only variable sites are reported. Arrows mark sites carrying insertions/deletions.

optimal by Modelstest was HKY+I+G with the following ML parameters: base frequencies were 0.2312, 0.2582, and 0.1976; *Ti/tv* = 2.7107; α = 0.5855; and the proportion of invariable sites was 0.4889. The position of *D. cuneata* varied depending on the analysis performed. In the ML tree it appeared more linked to *S. senegalensis/S. vulgaris* than to *S. kleinii* and *S. lascaris*; in the case of the MP tree, it was found forming part of the clade *Monochirus/Microchirus/D. hexophthalma* as the most basal taxon. Nevertheless, neither of these 2 switchings was supported by sufficient bootstrap values. For partial rRNA 16S gene, the best-fit ML model was also HKY+I+G. The ML parameters generated by Modeltest were as follows: base frequencies were 0.3223, 0.2564, and 0.1876; *Ti/tv* = 3.0250; α = 0.9504; and the proportion of invariable sites was 0.6079. The topology of MP and NJ phylogenetic trees constructed on rRNA 16S partial sequences gave the same results as those of the combined data trees, but some relationships appeared to be highly inconsistent in the ML tree: that is, a grouping of *S. lusitanica* with the *Solea/D. cuneata* lineage was observed, although the level of accuracy shown by this reconstruction was not significant (less than 50% of bootstrap replicates). Finally, in the case of rRNA 12S gene, the appropriate model of sequence evolution determined by Modeltest was TrN+G, with the following ML parameters: base frequencies were 0.3356, 0.2721, and 0.1845; rate matrix R(b) = 5.1932, R(e) = 12.1458; α = 0.1734; and the proportion of invariable sites was 0. The ML phylogenetic tree showed an evolutionary pattern also similar to that of the combined data, although a switching of positions was observed between *S. lascaris* and *S. lusitanica*. This unreliable relationship was not supported by significant bootstrap values. In fact, only the high-level relationship between *Monochirus*, *Microchirus*, and *D. hexophthalma* was consistent (75% of bootstrap replicates) in this tree. In MP and NJ rRNA 12S trees, *D. cuneata* was more related to *S. senegalensis/S. vulgaris* than to *S. kleinii* and *S. lascaris*. Yet each of these switchings was supported by less than 50% of bootstrap values. So, it is noteworthy that none of the relationships differing from the phylogenetic reconstructions obtained using the concatenated sequences were supported by bootstrap values higher than 50%.

An additional phylogenetic analysis was also performed: a NJ tree was constructed based on *cytb* amino acid sequences (Figure 4). The topology of the tree obtained was almost the same as that of combined data set, except that *S. lascaris* and *S. kleinii* formed a monophyletic group.

Table 3. Base Composition (in percentage) of Sole Species rRNA 12S (above diagonal), *cytb*, rRNA 16S sequences. For *cytb*, frequencies are given at each codon position. The bias has been calculated according to Irwin et al. (1991)

	Cytb											
	First				Second				Third			
Taxa	T	C	A	G	T	C	A	G	T	C	A	G
Solea lascaris	24.5	25.3	22.4	27.9	40.3	26.6	19.7	13.4	22.4	41.3	24.2	12.1
Solea senegalensis	23.9	24.7	23.7	27.6	40.5	26.1	19.5	13.9	21.6	41.1	30.5	6.8
Solea vulgaris	24.2	24.5	25.3	26.1	41.1	26.3	18.9	13.7	24.2	36.8	33.4	5.5
Solea kleinii	23.7	26.3	23.9	26.1	41.3	25.8	18.9	13.9	22.6	38.7	29.7	8.9
Microchirus azevia	22.4	26.1	22.9	28.7	39.7	27.4	20.0	12.9	19.2	45.3	28.9	6.6
Microchirus boscanion	22.1	25.5	25.5	26.8	40.8	26.1	19.7	13.4	23.4	41.6	27.4	7.6
Monochirus hispidus	22.1	25.3	24.7	27.9	40.0	26.6	19.5	13.9	29.2	35.8	27.9	7.1
Dicologlossa cuneata	23.7	26.1	23.2	27.1	42.1	24.7	19.2	13.9	22.6	40.0	29.7	7.6
Dicologlossa hexophthalma	22.6	25.8	23.9	27.6	40.3	26.8	19.2	13.7	20.0	44.2	29.2	6.6
Synaptura lusitanica	22.4	26.6	27.1	23.9	42.1	23.9	20.5	13.4	26.8	35.5	29.2	8.4
Mean	23.2	25.6	24.3	27.0	40.8	26.0	19.5	13.6	23.2	40.0	29.0	7.7
SD	0.93	0.69	1.41	1.35	0.83	1.03	0.50	0.33	2.98	3.35	2.34	1.82
Bias	0.041				0.227				0.263			

	rRNA 12S					rRNA 16S				
Taxa	T	C	A	G		Taxa	T	C	A	G
Solea lascaris	22.6	27.6	29.5	20.3		*Solea lascaris*	23.7	24.8	29.2	22.3
Solea senegalensis	20.4	27.5	30.8	21.3		*Solea senegalensis*	22.1	26.2	29.9	21.7
Solea vulgaris	21.2	26.5	31.8	20.4		*Solea vulgaris*	22.5	26.8	29.5	21.2
Solea kleinii	21.0	28.1	31.0	19.9		*Solea kleinii*	22.0	27.1	30.0	20.9
Microchirus azevia	21.7	29.4	30.0	19.0		*Microchirus azevia*	23.3	26.1	29.0	21.6
Microchirus boscanion	21.9	29.7	31.2	17.1		*Microchirus boscanion*	23.5	25.1	28.2	23.1
Monochirus hispidus	21.8	27.7	31.8	18.7		*Monochirus hispidus*	23.3	26.0	28.7	22.0
Dicologlossa cuneata	21.6	27.1	31.5	19.9		*Dicologlossa cuneata*	22.9	25.2	29.5	22.4
Dicologlossa hexophthalma	21.5	28.3	30.4	19.8		*Dicologlossa hexophthalma*	22.8	26.3	29.8	21.1
Synaptura lusitanica	21.5	27.2	30.6	20.6		*Synaptura lusitanica*	22.6	25.4	29.8	22.2
Mean	21.5	27.9	30.9	19.7		Mean	22.9	25.9	29.4	21.9
SD	0.58	1.00	0.76	1.18		SD	0.58	0.76	0.58	0.68
Bias	0.117					Bias	0.071			

Discussion

Taxonomic classification of fishes based only on morphologic characters has been shown to be successful in defining species and in organizing these species into different genera. Yet meristic and morphologic classification should be revised using molecular techniques. Recent years have witnessed an explosion in phylogenetic studies based on molecular data (see Kocher and Stepien, 1997). Flatfishes are not an exception, and several surveys have focused on the relatedness of different families of Pleuronectiformes (Tinti et al., 1999; Berendzen and Dimmick, 2002) and among species included in the family Soleidae (Tinti and Piccinetti, 2000; Tinti et al., 2000). Phylogenetic analyses reported here complement these studies with the inclusion of new molecular data and new species.

The *cytb* gene is undoubtedly one of the most used protein-coding genes in phylogeny of fishes (Kocher and Stepien, 1997). Among soles, previous studies have employed only partial sequences of this gene to establish

Table 4. Pairwise Tamura-Nei's Genetic Distances of Soleid Species Calculated on *cytb*, rRNA 12S, and rRNA 16S Sequences

	S. lascaris	*S. senegalensis*	*S. vulgaris*	*S. kleinii*	*M. azevia*	*M. boscanion*	*M. hispidus*	*D. cuneata*	*D. hexophthalma*	*S. lusitanica*	*P. flesus*
Cytochrome *b* (rRNA 12S)											
Solea lascaris	—	0.103	0.085	0.088	0.137	0.165	0.149	0.088	0.135	0.248	0.206
Solea senegalensis	0.059	—	0.040	0.062	0.115	0.169	0.136	0.061	0.114	0.249	0.194
Solea vulgaris	0.068	0.038	—	0.068	0.127	0.159	0.143	0.056	0.124	0.235	0.202
Solea kleinii	0.064	0.064	0.067	—	0.109	0.157	0.130	0.061	0.108	0.226	0.196
Microchirus azevia	0.086	0.086	0.077	0.093	—	0.092	0.059	0.099	0.032	0.216	0.175
Microchirus boscanion	0.098	0.092	0.085	0.089	0.056	—	0.097	0.147	0.079	0.241	0.196
Monochirus hispidus	0.111	0.098	0.092	0.102	0.058	0.067	—	0.124	0.066	0.233	0.191
Dicologlossa cuneata	0.085	0.090	0.072	0.082	0.087	0.097	0.102	—	0.090	0.236	0.200
Dicologlossa hexophthalma	0.093	0.086	0.077	0.089	0.030	0.055	0.053	0.085	—	0.230	0.184
Synaptura lusitanica	0.129	0.132	0.126	0.132	0.141	0.146	0.144	0.150	0.139	—	0.245
Platichthys flesus	0.138	0.138	0.141	0.143	0.130	0.147	0.140	0.135	0.143	0.142	—
	S. lascaris	*S. senegalensis*	*S. vulgaris*	*S. kleinii*	*M. azevia*	*M. boscanion*	*M. hispidus*	*D. cuneata*	*D. hexophthalma*	*S. lusitanica*	*P. flesus*
rRNA 16S											
Solea lascaris	—										
Solea senegalensis	0.092	—									
Solea vulgaris	0.079	0.039	—								
Solea kleinii	0.089	0.071	0.054	—							
Microchirus azevia	0.084	0.098	0.072	0.079	—						
Microchirus boscanion	0.123	0.111	0.103	0.106	0.054	—					
Monochirus hispidus	0.105	0.096	0.081	0.106	0.036	0.061	—				
Dicologlossa cuneata	0.084	0.196	0.082	0.072	0.081	0.091	0.096	—			
Dicologlossa hexophthalma	0.096	0.106	0.082	0.094	0.028	0.061	0.057	0.093	—		
Synaptura lusitanica	0.201	0.181	0.193	0.199	0.184	0.184	0.187	0.185	0.188	—	
Platichthys flesus	0.192	0.182	0.186	0.174	0.169	0.155	0.179	0.164	0.173	0.209	—

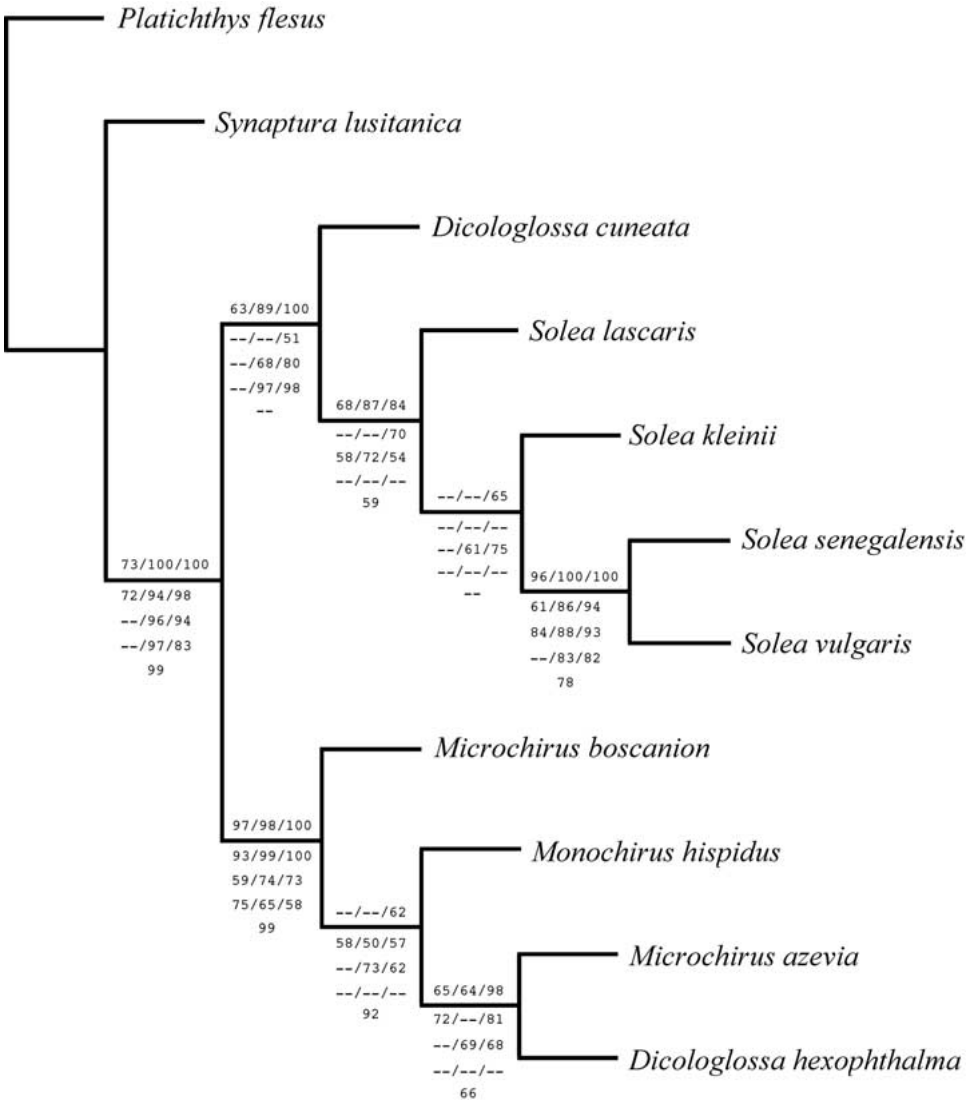

Figure 4. Phylogenetic relationships of sole species present in the Gulf of Cádiz. Maximum likelihood, maximum parsimony, and neighbor-joining bootstrap values higher than 50% are indicated for the concatenated sequence data set (above nodes) and for *cytb*, rRNA16S, and rRNA 12S individual nucleotide sequences, respectively (below nodes). Bootstrap values for the NJ tree based on *cytb* amino acid sequences are also shown.

phylogenetic relationships (Tinti and Piccinetti, 2000; Tinti et al., 2000; Borsa and Quignard, 2001), and some results have been incongruent with those obtained using rRNA 16S (Tinti et al., 2000). It has been shown that the use of limited sequence data may cause errors in estimates of evolutionary relatedness among taxa owing to a large variance in substitution rate. Hence, longer sequences are preferable, especially if we consider the restrictions in mutation imposed on protein-coding genes (Martin et al., 1990). This situation is still more complicated if the molecular marker (like *cytb*) evolves quickly, which can lead to the loss of phylogenetic information between distantly related taxa through homoplasy (Irwin et al., 1991; Meyer, 1994; Lydeard and Roe, 1997). In fact, we have found evidence of this phenomenon in *cytb*, with transitions appearing saturated relative to transversions. This might explain the incongruencies cited above. In this case it is advisable to infer phylogenetic relationships using genes with a slower substitution rate like rRNA mitochondrial genes (Orti, 1997; Stepien et al., 1997), or even better a combination of differently evolving genes (Sarver et al., 1996; Freshwater et al., 2000; Apostolidis et al., 2001). In the present study analyses were performed using a combined data set of *cytb* and rRNA mitochondrial genes. The strong correlation in the topology of the trees inferred by ML, MP, and NJ methods demonstrates the high level of accuracy of the phylogenetic reconstruction carried out in this survey.

Present analysis supports fully the phylogenetic relatedness of the *Solea*-like species *S. vulgaris* and *S. senegalensis*, which were the closest sister *Solea* species in all reconstructions. In regard to this issue, a series of morphologic characters is in agreement with this relatedness: anterior nostril on blind side not enlarged, without fringes,

and with a diameter almost equal to the length of scales on the body (Ben-Tuvia, 1990). The grouping of *S. vulgaris* and *S. senegalensis* inferred from our data is in complete agreement with previously published phylogenetic analyses based on partial nucleotide sequences of *cytb* and rRNA 16S mitochondrial genes (Tinti and Piccinetti, 2000). In contrast, it is interesting to note how the *Pegusa*-like species *S. kleinii* and *S. lascaris* do not appear as a monophyletic group, contrary to *Solea*-like species, except in the NJ tree based on *cytb* amino acid sequences. This result, in agreement with other published results (Tinti and Piccinetti, 2000; Tinti et al., 2000), suggests that the grouping of *S. kleinii* and *S. lascaris* into the *Pegusa* subgroup based on a shared enlarged nostril is not taxonomically appropriate.

This work provides the first molecular data for the species *D. hexophthalma*. It is noteworthy that in our analyses, surprisingly, the most related species was *M. azevia* instead of *D. cuneata*. The traditional existence of the genus *Dicologlossa* with 2 species (*D. cuneata* and *D. hexophthalma*) has been supported in the presence of a distinct supratemporal branch of the lateral line with an angular S shape (Quéro et al., 1986; Bauchot, 1987). However, in a taxonomic revision of soles from the eastern Atlantic and Mediterranean Sea based on 20 different biometric and osteologic features, Desoutter (1994) proposed the subdivision of the genus *Microchirus* in 2 subgenera: *Microchirus* and *Zevaia*. The former regroups 5 species, including *M. boscanion*. The subgenus *Zevaia* contains 2 species: *M. azevia* and *D. hexophthalma*. The inclusion of *D. hexophthalma* in the genus *Microchirus* is mainly based in the shape of the urohyal, which presents 2 distinct branches (dorsal and ventral) forming an acute angle. This shape is different enough in *D. cuneata* to justify the segregation of this species from the subgenus *Zevaia* (Desoutter, 1994). Our data and those of Desoutter support a taxonomic revision of the present status of these 2 species.

Another important issue to take in account is the closer relatedness of *M. azevia* with *M. hispidus* than to its congeneric *M. boscanion*. Similar results were obtained in a previous phylogenetic survey of soles based on partial sequences of *cytb* and rRNA 16S genes, with *Microchirus ocellatus* more linked to *M. hispidus* than to *Microchirus variegatus* (Tinti et al., 2000). In fact, in that survey these species appeared as a monophyletic group, and the authors argued against the separation of these 2 genera. The differentiation of *Microchirus* and *Monochirus* is morphologically based on the presence or absence of a reduced pectoral fin on the blind side (Quéro et al., 1986; Bauchot, 1987). Our results do not support the differentiation into 2 genera and indicate that the taxonomic relevance of this character is at least questionable.

In view of the results in total, it seems necessary to perform a more complete molecular analysis of most of the species of soles present in the eastern Atlantic and even the Mediterranean Sea for better resolution of the phylogenetic relationships among them. In this sense the disposal of longer sequences will help to clarify with more accuracy such relations. Nevertheless, the present study could serve as reference in attempts to resolve relationships between these lineages.

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References

Adachi, J., and Hasegawa, M. (1996). MOLPHY Version 2.3: Programs for Molecular Phylogenetics Based on Maximum Likelihood. Computer Science Monographs No. 28. Tokyo, Japan: The Institute for Statistical Mathematics.

Apostolidis, A.P., Mamuris, Z., and Triantaphyllidis, C. (2001). Phylogenetic relationships among four species of Mullidae (Perciformes) inferred from DNA sequences of mitochondrial cytochrome *b* and 16S rRNA genes. *Biochem Syst Ecol* 29:901–909.

Bauchot, M.L. (1987). Poissons osseux. In: *Fiches FAO d'identification pour les besoins de la pèche. (rev. 1). Méditerranée et mer Noire. Zone de pêche 37, Vol II*, Fisher, W., Bauchot, M.L., and Schneider, M. (eds.). Rome, Italy: Commission des Communautés Européennes and FAO, pp 891–1421.

Ben-Tuvia, A. (1990). A taxonomic reappraisal of the Atlanto-Mediterranean soles *Solea solea*, *S. senegalensis* and *S. lascaris*. *J Fish Biol* 36:947–960.

Berendzen, P.B., and Dimmick, W.W. (2002). Phylogenetic relationships of Pleuronectiformes based on molecular evidence. *Copeia* 3:642–652.

Bini, G. (1968). Atlante del pesci delle coste italiane: Osteitti, Vol. 8. Rome, Italy: Mondo Sommerso.

Borsa, P., and Quignard, J.P. (2001). Systematics of the Atlantic-Mediterranean soles *Pegusa impar*, *P. lascaris*, *Solea aegyptiaca*, *S. senegalensis*, and *S. solea* (Pleuronectiformes: Soleidae). *Can J Zool* 79:2297–2302.

Borsa, P., Blanquer, A., and Berrebi, P. (1997). Genetic structure of the flounders *Platichthys flesus* and *P. stellatus* at different geographic scales. *Mar Biol* 129:233–246.

Chapleau, F. (1993). Pleuronectiform relationships: a cladistic reassessment. *Bull Mar Sci* 52:516–540.

Cooper, J.A., and Chapleau, F. (1998a). Monophyly and intrarelationships of the family Pleuronectidae (Pleuronectiformes), with a revised classification. *Fish Bull* 96:686–726.

Cooper, J.A., and Chapleau, F. (1998b). Phylogenetic status of *Paralichthodes algoensis* (Pleuronectiformes: Paralichthodidae). *Copeia* 2:477–481.

Cunningham, C.W., Zhu, H., and Hillis, D.M. (1998). Best-fit maximum likelihood models for phylogenetic inference: empirical tests with known phylogenies. *Evolution* 52:978–987.

Desoutter, M. (1994). Revison of the genera *Microchirus*, *Dicologlossa* and *Vanstraelenia* (Pleuronectiformes, Soleidae). *Cybium* 18:215–249.

Exadactylos, A., and Thorpe, J.P. (2001). Allozyme variation and genetics inter-relationships between seven flatfish species (Pleuronectiformes). *Zool J Linn Soc* 132:487–499.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791.

Felsenstein J. (2004). PHYLIP (Phylogeny Inference Package) Version 3.6b. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Freshwater, D.W., Khyn-Hansen, C., Sarver, S.K., and Walsh, P.J. (2000). Phylogeny of *Opsanus* spp. (Batrachoididae) inferred from multiple mitochondrial-DNA sequences. *Mar Biol* 136:961–968.

Hedges, S.B. (1992). The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies. *Mol Biol Evol* 9:366–369.

Hensley, D.A. (1997). An overview of the systematics and biogeography of the flatfishes. *J Sea Res* 37:187–194.

Hoshino, K. (2001). Monophyly of the Citharidae (Pleuronectoidei: Pleuronectiformes: Teleostei) with considerations of pleuronectoid phylogeny. *Ichthyol Res* 48:391–404.

Hoshino, K., and Amaoka, K. (1998). Osteology of the flounder, *Tephrinectes sinensis* (Lacepède) (Teleostei: Pleuronectiformes), with comments on its relationships. *Ichthyol Res* 45:69–77.

Irwin, D.M., Kocher, T.D., and Wilson, A.C. (1991). Evolution of the cytochrome *b* gene of mammals. *J Mol Evol* 32:128–144.

Kocher, T.D., and Stepien, C.A. (1997). Molecular Systematics of Fishes. San Diego, Calif.: Academic Press.

Kotoulas, G., Bonhomme, F., and Borsa, P. (1995). Genetic structure of the common sole *Solea vulgaris* at different geographic scales. *Mar Biol* 122:361–375.

Lydeard, C., and Roe, K.J. (1997). The phylogenetic utility of the mitochondrial cytochrome *b* gene for inferring relationships among Actinopterygian fishes. In: *Molecular Systematics of Fishes*, Kocher, T.D., and Stepien, C.A. (eds.). San Diego, Calif.: Academic Press, pp 285–303.

Martin, A.P., Kessing, B.D., and Palumbi, S.R. (1990). Accuracy of estimating genetic distance between species from short sequences of mitochondrial DNA. *Mol Biol Evol* 7:485–488.

Meyer, A. (1994). Shortcomings of the cytochrome *b* gene as a molecular marker. *Tree* 9:278–280.

Orti, G. (1997). Radiation of Characiform fishes: evidence from mitochondrial and nuclear DNA sequences. In: *Molecular Systematics of Fishes*, Kocher, T.D., and Stepien, C.A. (eds.). San Diego, Calif.: Academic Press, pp 219–243.

Posada, D., and Crandall, K.A. (1998). Modeltest: testing the model of DNA substitution. Bioinformatics 14 (in press)

Quéro, J.C., Desoutter, M., and Lagardere, F. (1986). Soleidae. In: *Fishes of the North eastern Atlantic and the Mediterranean, Vol 3*, Whitehead, P.J., Bauchot, M.L., Hureau, J.C., Nielsen, J., and Tortonese, E. (eds.). Paris, France: UNESCO, pp 1308–1328.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.

Sarver, S.K., Freshwater, D.W., and Walsh, P.J. (1996). Phylogenetic relationships of western Atlantic snappers (family Lutjanidae) based on mitochondrial DNA sequences. *Copeia* 3:715–721.

Stepien, C.A., Dillon, A.K., Brooks, M.J., Chase, K.L., and Hubers, A.N. (1997). The evolution of Blennioid fishes based on an analysis of mitochondrial 12S rDNA. In: *Molecular Systematics of Fishes*, Kocher, T.D., and Stepien, C.A. (eds.). San Diego, Calif.: Academic Press, pp 245–270.

Swofford, D.L. (2000). PAUP* Phylogenetic Analysis Using Parsimony (*and other methods) Version 4.0b2. Sunderland, Mass.: Sinauer.

Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 10:512–526.

Tinti, F., and Piccinetti, C. (2000). Molecular systematics of the Atlanta-Mediterranean *Solea* species. *J Fish Biol* 56:604–614.

Tinti, F., Colombari, A., Vallisneri, M., Piccinetti, C., and Stagni, A.M. (1999). Comparative analysis of a mitochondrial DNA control region fragment amplified from three Adriatic flatfish species and molecular phylogenesis of Pleuronectiformes. *Mar Biotechnol* 1:20–24.

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

Torchio, M. (1973). Soleidae. In: *Check-list of the Fishes of the North-Eastern and of the Mediterranean*, Hureau, J.C., and Monod, T. (eds.). Paris, France: UNESCO, pp 628–634.

Tortonese, E. (1975). Osteichthyes (Pesci ossei). In: *Fauna d'Italia, XI*, Bologna, Itlay: Calderini, pp 479–523.

Verneau, O., Moreau, C., Catzeflis, F.M., and Renaud, F. (1994). Phylogeny of flatfishes (Pleuronectiformes): comparisons and contradictions of molecular and morphoanatomical data. *J Fish Biol* 45:685–696.