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Systematics of the Atlantic-Mediterranean soles *Pegusa impar*, *P. lascaris*, *Solea aegyptiaca*, *S. senegalensis*, and *S. solea* (Pleuronectiformes: Soleidae)

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Abstract: Nucleotide sequence variation at the cytochrome *b* locus was investigated in five *Solea* species, together with a reappraisal of meristic data and a review of allozyme data pertinent to their systematics. *S. aegyptiaca*, considered a synonym of *S. solea*, and *S.* (*Pegusa*) *impar*, considered a synonym of *S.* (*P.*) *lascaris*, are shown to be valid species according to the morphological, phylogenetic, genotypic, and biological species definitions. The validity of the genus *Pegusa* was examined in the light of both allozyme and cytochrome *b* gene sequence data.

Résumé : Les séquences nucléotidiques au locus du cytochrome *b* ont été analysées chez cinq espèces du genre *Solea*, et conjointement une ré-évaluation des données méristiques et une synthèse des données génétiques pertinentes à leur systématique ont été faites. La distinction entre *S. aegyptiaca* et *S. solea*, ainsi que celle entre *S.* (*Pegusa*) *impar* et *S.* (*P.*) *lascaris*, toutes deux ayant été remises en cause dans la littérature, sont pourtant en plein accord avec les définitions morphologique, phylogénétique, génotypique et biologique de l'espèce. La validité du genre *Pegusa* est discutée à la lumière des données allozymiques et des données de séquence au locus du cytochrome *b*.

Introduction

Seven nominal species have been recognised in the genus *Solea* in the northeastern Atlantic and the Mediterranean (*S. aegyptiaca*, *S. impar*, *S. kleini*, *S. lascaris*, *S. nasuta*, *S. senegalensis* and *S. vulgaris*; Quéro et al. 1986). *S. aegyptiaca* Chabanaud, 1927 was considered to be a species distinct from *S. solea* (Linnaeus, 1758) [=*S. vulgaris* Quensel, 1806; see Wheeler 1988]. This distinction followed a partial revision of the genus based on meristics and allozyme electrophoresis of samples from the Golfe-du-Lion in the Western Mediterranean and the Khalîj-Qâbis in the Eastern Mediterranean (Quignard et al. 1984). Ben Tuvia (1990) synonymised *Solea aegyptiaca* with *S. solea* because some morphometric characters (numbers of anal fin rays, dorsal fin rays, and vertebrae), earlier reported to be different between the two taxa (Chabanaud 1927; Quignard et al. 1984), were overlapping. Ben Tuvia (1990) considered that variation in the number of vertebrae, the only character previously quoted as diagnostic between the two species (Quignard et al. 1984), "can be attributed to the differences in hydrographic conditions at the time of spawning in various geographical regions". Ben Tuvia (1990) also synonymised *Solea impar* Bennett 1831 and *S. nasuta* (Pallas, 1811) under *S. lascaris* (Risso, 1810), on the basis that insufficient diagnostic characters had been given by previous authors to enable their separation.

Electrophoretic studies (Quignard et al. 1984; Pasteur et al. 1985; Goucha et al. 1987; She et al. 1987a, b) however have demonstrated that *S. aegyptiaca* and *S. solea* are reproductively isolated from each other wherever they were found in sympatry, *i.e.* in the Golfe-du-Lion, along the coast of Tunisia, and in the Suez Canal. Allozymes also revealed that between *S. impar* and *S. lascaris* alternative alleles are fixed at a considerable proportion of loci (9/20; Goucha et al. 1987) thus demonstrating their genetic isolation. The separation of *S. solea* from *S. aegyptiaca*, and that of *S. impar* from *S. lascaris* were further supported by a phylogenetic tree inferred from allozymes (Goucha et al. 1987). Extant hybridisation was reported between *S. aegyptiaca* and *S. senegalensis* Kaup 1858 (She et al. 1987a).

Tinti and Piccinetti (2000) examined nucleotide variation at two mitochondrial-DNA loci (*16S rRNA*, *cytochrome b*) in *Solea* spp. samples from the Mediterranean, with the aim "to provide an independent insight into the systematics of molecular characters which, with respect to the morphological ones, are free from subjective interpretations and environmental pressure". Surprisingly, the mitochondrial-DNA sequences of their "*S. aegyptiaca*" sample appeared to be very close to the sequences of their *S. solea* sample. These authors thus endorsed the synonymy of *S. aegyptiaca* with *S. solea*, and also that of *S. impar* with *S. lascaris*, because of the close molecular relatedness of individuals presumed to be *S. lascaris* with *S. impar* (1.6% and 0.3% nucleotide divergence at the *16S rRNA* and *cytochrome b* loci, respectively). Tinti and Piccinetti (2000) also sampled in the Ionian Sea soles "with ambiguous characters" they eventually referred to as "*S. senegalensis*", with 39-41vertebrae, and whose mitochondrial-DNA sequences were distant from those of Cádiz-Bay *S. senegalensis* by 3.8% (16S rDNA) and 11.6% (cytochrome *b* gene) nucleotide divergence.

Altogether, morphometrics, allozymes, and mitochondrial-DNA phylogenies thus have been used to support apparently contradictory views of the systematics and taxonomy of *Solea* species. The aim of this note is to clarify the systematic relationships among Atlanto-Mediterranean *Solea* species. For this, we reassessed Ben Tuvia's (1990) results, compiled and analysed a comprehensive allozyme dataset from the literature, and added new phylogenetic information to that provided by Tinti and Piccinetti (2000) by analysing nucleotide variation at the cytochrome *b* locus in new samples of *S. aegyptiaca*, *S. lascaris*, and *S. solea*. In addition, the genetic data allowed to test the validity of a distinct genus *Pegusa* grouping *S. impar*, *S. lascaris* and *S. nasuta* vs. other *Solea* spp (e.g. Bini 1968).

Materials and Methods

To examine the extent of genetic differences between species relative to the variation within species, we compiled allozyme data on *Solea* spp. populations from Quignard et al.

(1984), Goucha et al. (1987), She et al. (1987a, b), and Kotoulas et al. (1995). All the foregoing studies were conducted in the same laboratory, using the same protocols, thus making cross-comparisons straightforward. The electromorph frequencies at 5 enzyme loci (namely *Aat-2*, *Gpi-1*, *Gpi-2*, *Ldh-2*, *Pt-3*) scored in common in all studies and in five Atlanto-Mediterranean *Solea* species were arranged under a matrix form suitable for correspondence analysis (Lebreton et al. 1990).

The new material analysed for nucleotide variation at the cytochrome *b* locus consisted of 16 *Solea aegyptiaca* sampled May 2000 in Zarzis, Tunisia [33°28'N, 11°07'E], 8 *S. solea* from Pertuis Breton, France [46°19'N, 01°24'W], November 1999, 2 *S. solea* from an unknown location on the Atlantic coast of France, June 2000, 4 *S. lascaris* from the Loire estuary, France [47°06'N, 02°20'W], June 2000, and 7 *S. lascaris* from Pertuis Charentais, France [45°48'N, 01°14'W], June 2000. The samples were identified to species according to the identification key provided by Quéro et al. (1986). The numbers of dorsal fin rays in *S. aegyptiaca* (mean±*SD*=71.8±1.3; *N*=13) and in *S. solea* (81.1±1.7; *N*=10) were in accordance with previous reports (Quignard et al. 1984; Quéro et al. 1986). The DNA of each individual was extracted using phenol-chloroform-isoamylalcohol, and a 354 base-pair portion of the cytochrome *b* gene was amplified by polymerase chain reaction (PCR) using universal primers for the *CB2-H*/*CB1-L* fragment (see Palumbi et al. 1991) as did Tinti and Piccinetti (2000). The PCR products were formamide-denaturated to single DNA strands and subjected to electrophoresis on non-denaturing polyacrylamide gel (SSCP), thus revealing nucleotide-sequence polymorphism, as in Hoarau and Borsa (2000). All SSCP variants (two in 16 *S. aegyptiaca*, one in 11 *S. lascaris*, and two in 10 *S. solea*) were sequenced using the Thermosequenase kit (Amersham Life Science, Cleveland OH, USA) with ^{33}P-labelled dideoxynucleotides (Amersham). All five sequences, which were deposited in GenBank (accession nos. AF289716-289720), were aligned on 301 bp with all other *Solea* spp. cytochrome *b* gene sequences in GenBank.

A phylogenetic tree was derived from the matrix of nucleotide divergence estimates among sequences using the Neighbor-Joining algorithm; nucleotide divergences were estimated using Kimura's 2-parameter model with a ratio of two transitions to one transversion (procedures DNADIST and NEIGHBOR of PHYLIP; Felsenstein 1993). The robustness of the nodes was tested by 1000 bootstraps resamplings of the sequence matrix using procedure SEQBOOT of PHYLIP. Parsimony analysis was done on the same sequence dataset using the MAXIMUM PARSIMONY procedure of MEGA (Kumar et al. 1993), with 1000 bootstrap resamplings. The Soleidae *Microchirus variegatus* (Donovan, 1808) was chosen as outgroup because of its genetic distance with any *Solea* species being larger than interspecies genetic distances within the genus *Solea* (Goucha et al. 1987; Tinti et al. 2000). A nuclear phylogeny was also inferred using the Neighbor-joining algorithm on the matrix of pairwise Nei's genetic distances between species (procedures GENDIST, NEIGHBOR and SEQBOOT of PHYLIP). Nei's genetic distances were based on allozyme frequency data at 16 loci, that is all loci scored by Goucha et al. (1987) except *Ck* which was also scored as locus *Pt-3* (P. Borsa pers. obs.).

The systematic positions of *S. kleini* (Bonaparte, 1833) and *S. nasuta* were not addressed here because no sample material and no allozyme data for these species were available to us. However, partial nucleotide sequences of the cytochrome *b* gene and 16S rDNA of *S. kleini* have been presented by Tinti and Piccinetti (2000).

A subsample of the fish analysed in the present study was deposited as voucher specimens at Museum National d'Histoire Naturelle (MNHN), Paris under registration nos. MNHN 2000-5629 to 5633 (*S. aegyptiaca*), MNHN 2000-5637 to 5640 (*S. solea*), and MNHN 2000-5634 to 5636 (*S. lascaris*). In the absence of any known holotype or paratype, *S. solea* specimen no. MNHN 2000-5637 was designated as neotype, in conformity with the recommendations of the International Commission on Zoological Nomenclature (1999). The designation of an historical specimen collected by P. Chabanaud and preserved at MNHN as neotype for *S. aegyptiaca* is pending (J.-C. Hureau, in litt.).

Results and Discussion

Although the distinction of *Solea aegyptiaca* from *S. solea* on the basis of meristic characters (Quignard et al. 1984) was deemed unreliable by Ben Tuvia (1990), the numbers of vertebrae, dorsal fin rays, and anal fin rays presented in the latter article for "*Solea solea*" (Ben Tuvia's tables II, III and IV) had bimodal distributions. For any of these characters, each mode of the distribution corresponded to the mode previously given for either *S. aegyptiaca* or *S. solea* (Quignard et al. 1984), and the degree of variation within either species throughout the Mediterranean was lower than between species at any given location (Quignard et al. 1984). The distribution of the number of vertebrae presented for "*S. lascaris*" by Ben Tuvia (1990: table VI) was bimodal, with the first mode corresponding to typical *S. impar* samples and the second mode corresponding to samples collected in the northeastern Atlantic where the predominant species is *S. lascaris* (Marinaro 1988; J.-P. Quignard pers. obs.). The distinction between *S. impar* and *S. lascaris* was also evident from the distributions of the numbers of dorsal fin and anal fin rays compared to the values given for either of the type specimens examined by Ben Tuvia [table VII of Ben Tuvia (1990)]. Numbers of vertebrae plotted against numbers of either anal fin rays or dorsal fin rays for individual "*Pegusa lascaris*" from the Atlantic and the Mediterranean [including *S. impar* and *S. lascaris*, then considered as synonyms; see figures 35 and 36 of Chabanaud (1929)] provided even more convincing evidence of two distinct morphs, as two disjunct clusters were observed on each scattergram.

Correspondence analysis of allozyme-frequency data (Fig. 1) showed the total separation of each taxon relative to the others. *S. aegyptiaca*, *S. senegalensis*, and *S. solea* were each represented by 4-5 samples collected across wide geographical areas. For instance, all *S. solea* samples, including samples from Brittany (*A*), Golfe-du-Lion (*B*, *C*) and Suez (*EG*) clustered onto a small spot on Fig. 1. Indeed, geographic differentiation in *S. solea* is weak albeit detectable, with pairwise F_{ST} (Wright 1951) estimates increasing by only ca. 0.01 every 1000 km in an isolation-by-distance fashion from the English Channel to the Eastern Mediterranean (Kotoulas et al. 1995; Borsa et al. 1997b). Such low levels of genetic heterogeneity across vast distances in each of these three taxa, and their clear separation from one another (Fig. 1) warrants their recognition as separate species, in spite of hybridisation in areas of contact (between *S. aegyptiaca* and *S. senegalensis*; She et al. 1987a).

The most common mitochondrial-DNA haplotype (cytochrome *b* gene) found by us in Atlantic *Solea solea* (GenBank AF289716; frequency=0.90) was identical to the apparently most common *S. solea* haplotype found in the Adriatic Sea by Tinti and Piccinetti (2000) (GenBank AF113181=AF113184=AF11185). The other haplotype we found in Atlantic *S. solea* (GenBank AF289717; frequency=0.10) differed from the former by one nucleotide transition. The most common haplotype in *S. aegyptiaca* from southern Tunisia (GenBank AF289718; frequency=0.94) was identical to one of the two haplotypes found by Tinti and Piccinetti in Ionian-Sea *Solea* sp. specimens (eventually referred to as "*S. senegalensis*"; GenBank AF113188=AF113190=AF113191). The rarer *S. aegyptiaca* haplotype (GenBank AF289719; frequency=0.06) differed from the former by one nucleotide transition. Knowing that *S. senegalensis* hybridises with *S. aegyptiaca* in a narrow zone of contact in northern Tunisia (She et al. 1987a), one cannot exclude that *S. senegalensis* might be introgressed by *S. aegyptiaca* mitochondrial DNA. However, considering that *S. senegalensis* has not been reported from the Eastern Mediterranean (Quignard et al. 1986), and that the number of vertebrae of the specimens collected by Tinti and Piccinetti (2000) in the Ionian Sea were typical of *S. aegyptiaca* (Quignard et al. 1984; 1986), we here reassign Tinti and Piccinetti's Ionian-Sea *Solea* sp. sample to *S. aegyptiaca*. Finally, the unique haplotype found in *S. lascaris* (GenBank AF289720) differed from *S. impar* (GenBank AF113194) by 6.0% nucleotide change. The Ionian-Sea "*S. lascaris*" sequence provided by Tinti and Piccinetti (GenBank AF113195) was therefore much more closely related to *S. impar* than to Atlantic *S. lascaris*. The phylogeny presented in Fig. 2, inferred from nucleotide divergence estimates, demonstrated the clear separation of *S. solea* haplotypes from those of *S. aegyptiaca*, of *S. aegyptiaca* from *S. senegalensis*, and of *S. lascaris* from *S. impar*. The topology of the parsimony tree was identical to that of the Neighbor-Joining tree and was supported by high bootstrap scores (Fig. 2). The phylogenetic relationships of *S. aegyptiaca*, *S. senegalensis*, and *S. solea* cytochrome *b* gene sequences appeared to be similar to those inferred from electromorph-frequency data

(Goucha et al. 1987; Fig. 3), thus yielding no support to the *ad hoc* hypothesis that *S. senegalensis* possess *S. aegyptiaca* mitochondria.

Thus, the distinction between *S. solea* and *S. aegyptiaca* fulfills the morphological species definition (Quignard et al. 1984; Ben Tuvia 1990), the phylogenetic species definition (Goucha et al. 1987; present results), and the biological species definition since these two taxa are reproductively isolated throughout their range (Quignard et al. 1984; She et al. 1987b; present results); the data of She et al. (1987b) also conform to the genotypic-cluster definition of species (Mallet 1995). The distinction between *S. impar* and *S. lascaris* likewise fulfills the morphological and phylogenetic species definitions (Goucha et al. 1987; Ben Tuvia 1990; present results). The congruence of phylogenetic relationships derived from such independent datasets as allozymes and cytochrome *b* gene sequences is a both powerful and robust test of their systematics. These results restore the taxonomy of Quéro et al. (1986) and demonstrate that at the level of differentiation reached by the species in the genus *Solea*, mt-DNA or allozymes can equally be used as reliable characters for identification. Meristic characters effectively distinguish *S. aegyptiaca* from *S. solea* and *S. impar* from *S. lascaris*; however the assignment of a small proportion of individuals may be ambiguous when using a single character.

Chabanaud (1927) has suggested that *Solea impar* and *S. lascaris* be grouped into the genus *Pegusa* Günther, 1862 *sensu stricto*, on the basis of shared morphological features which are absent in the other Atlantic-Mediterranean *Solea* species (except *S. nasuta*), such as anterior nostril on blind side enlarged, rosette-shaped, and close to posterior nostril. Bini (1968) and Desoutter (1990) again include *S. impar*, *S. lascaris* and *S. nasuta* into the genus *Pegusa*. The mitochondrial tree (Fig. 2) yields some support to this distinction by grouping *S. impar* with *S. lascaris* as a separate clade, by the same time suggesting that the anterior nostril's shape and position are characters of phylogenetic value. As shown in the following, the case for considering these as a different genus is strong (see point 3) although not watertight (see points 1 and 2). **1** The Neighbour-Joining tree derived from the matrix of Nei's (1972) genetic distances based on 16 allozyme loci scored in all *S. aegyptiaca*, *S. impar*, *S. lascaris*, *S. senegalensis* and *S. solea*, and rooted by *Microchirus variegatus* [frequency data in Goucha et al. (1987)] had a step-like topology (Fig. 3). This tree did not exhibit separate clades for *S. aegyptiaca*, *S. senegalensis* and *S. solea* vs. *S. impar* and *S. lascaris* (both *Pegusa*). Instead, the strongest node, which was supported by a bootstrap score of 74%, distinguished a clade formed by *S. aegyptiaca* and *S. senegalensis* from all the other *Solea* species included in the analysis. **2** The average Nei's genetic distance between *S. impar* or *S. lascaris* and the other three *Solea* species was 1.28 (range 1.02 to 1.60). Although high, such values are not exceptional among species within a genus (Thorpe 1982). Such examples among marine Teleosteans include scad mackerels *Decapterus* spp. where Nei's genetic distances among species range from 0.49 to 1.52 (Kijima et al. 1988) and warehous *Seriolella* spp. (0.52 to 1.23; Bolch et al. 1994), but interspecific genetic distance estimates within a genus generally prove lower, e.g. in mullets *Liza* spp. (0.29 to 0.48; Autem and Bonhomme 1980), tunas *Thunnus* spp. (0.08 to 0.24; Elliott and Ward 1995), oreos *Neocyttus* spp. (0.10 to 0.12; Lowry et al. 1996), flounders *Platichthys* spp. (0.16 to 0.32; Borsa et al. 1997a), poor cods and bib *Trisopterus* spp. (0.63 to 0.82; Mattiangelli et al. 2000), etc. **3** Estimates of nucleotide divergence between haplotypes at the *cytochrome b* locus within a genus offer another yardstick to address the question. The estimates of nucleotide divergence between *S. impar* or *S. lascaris* haplotypes and those of the other *Solea* species ranged from 22.9% to 25.1%, averaging 23.9%. Using the same *CB2-H*/*CB1-L* fragment of the cytochrome *b* gene, this appeared to be significantly higher than in other genera e.g. *Beryx* spp. (range 4.8% to 8.5%; Hoarau and Borsa 2000), *Centroberyx* spp. (range 6.9% to 12.3%; sequences in Hoarau 1999), *Decapterus* spp. (range 10.1% to 17.2%; Perrin and Borsa, in press), but more of the same order as distance estimates between closely related genera e.g. *Beryx* spp. vs. *Centroberyx* spp. (range 9.0% to 15.7%; sequences in Hoarau 1999) or *Decapterus* spp. vs. *Selar crumenophthalmus* (range 19.1% to 26.2%; Perrin and Borsa, in press). To our view this is a sufficient argument in support of a distinct genus, namely *Pegusa*.

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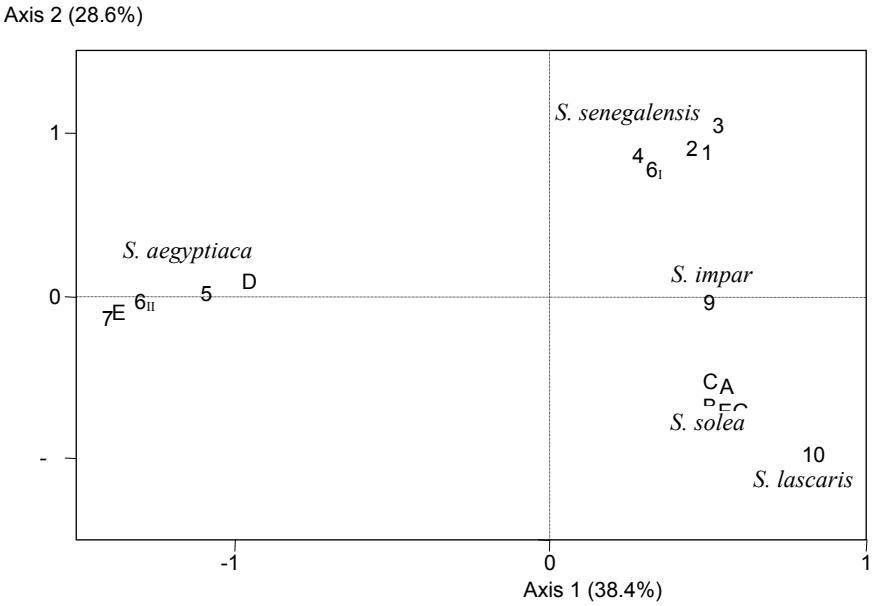

Fig. 1. Correspondence analysis (BIOMECO package; Lebreton et al. 1990): projection on the plane defined by Axis 1 and Axis 2 (percentages of total inertia in brackets) of 16 Atlanto-Mediterranean *Solea* spp. samples. All samples (*A*: Brittany; *B*, *C*, *D*, *E*, *5*, *9*: Golfe-du-Lion, Western Mediterranean; *EG*: Suez canal; *1*: Dakar, Senegal; *2*: Lisbon, Portugal; *3*: Ebro delta, Spain; *4*: Bizerte lagoon, Tunisia; 6_I, 6_{II}: Gulf of Tunis, Tunisia; *7*: Khalîj-Qâbis, Eastern Mediterranean; *10*: Brittany) were characterised by their electromorph frequencies at 5 allozyme loci (*Aat-2*, *Gpi-1*, *Gpi-2*, *Ldh-2*, and *Pt-3*; nomenclature according to Quignard et al. 1984). Data for samples *A-E* were from Quignard et al. (1984); *1-5*, 6_I, 6_{II} and *7*, from She et al. (1987a); *9-10*, from Goucha et al. (1987); *EG*, from Kotoulas et al. (1995).

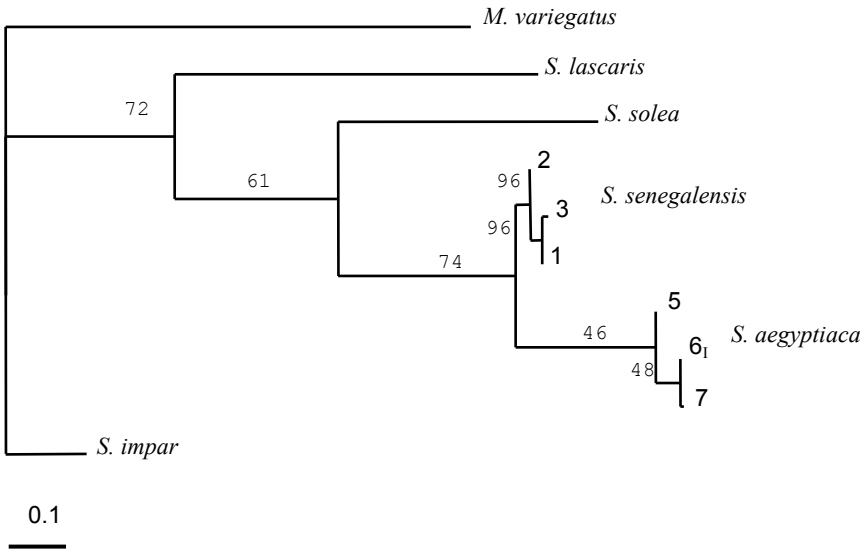

Fig. 3. Neighbor-joining tree (NEIGHBOR procedure in the PHYLIP package; Felsenstein, 1993) derived from the matrix of pairwise Nei's (1972) genetic distances among 5 Atlanto-Mediterranean *Solea* species. Nei's distances were calculated using the GENDIST procedure of PHYLIP, from electromorph-frequency data at 16 enzyme loci (*Aat-1*, *Aat-2*, *Aat-4*, *Est-1*, *Est-3*, *Glo*, *Gpd-1*, *Gpi-1*, *Gpi-2*, *Ldh-1*, *Ldh-2*, *Mdh-1*, *Pgm*, *Pt-3*, *Pt-4*, and *Sod-1*) scored in common in all 5 species and in outgroup *Microchirus variegatus* by Goucha *et al.* (1987). Numbers at a node are percentages of occurrence after bootstrap resampling of loci (1000 bootstraps) using procedure SEQBOOT of PHYLIP. Scale bar=0.1 Nei's genetic distance.

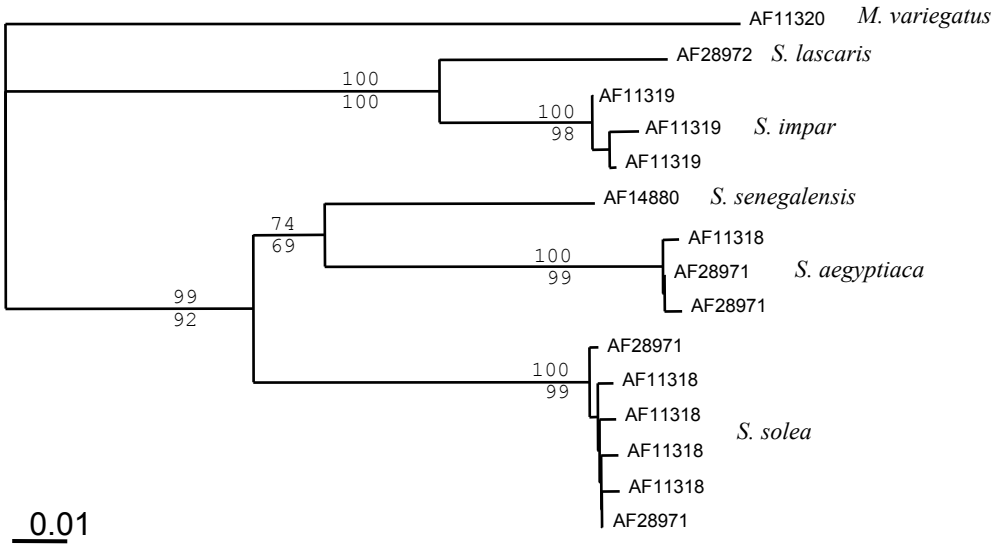

Fig. 2. Neighbour-joining tree (NEIGHBOR procedure in the PHYLIP package; Felsenstein 1993) of partial nucleotide sequences (301 bp) of the cytochrome *b* gene in 5 Atlanto-Mediterranean *Solea* species, using *Microchirus variegatus* as outgroup. Sequence numbers are those allocated by GenBank. Numbers at a node are percentages of occurrence after 1000 bootstrap resamplings of nucleotide sites (Neighbor-Joinig bootstrap values above branches, parsimony values below branches). Unlabelled nodes had bootstrap scores <74%. Scale bar=1% nucleotide divergence.