NOTE

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, with a reappraisal of meristic data and a review of allozyme data pertinent to their systematics. *Solea aegyptiaca*, considered a synonym of *Solea solea*, and *Solea* (*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 *Solea aegyptiaca* et *Solea solea*, ainsi que celle entre *Solea* (*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 examinée à la lumière des données allozymiques et des données sur les séquences au locus du cytochrome *b*.

Introduction

Seven nominal species have been recognised in the genus *Solea* in the northeastern Atlantic Ocean and Mediterranean Sea (*S. aegyptiaca*, *S. impar*, *S. kleini*, *S. lascaris*, *S. nasuta*, *S. senegalensis* and *S. vulgaris*; Quéro et al. 1986). *Solea 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) synonymized *S. aegyptiaca* with *S. solea* because some morphometric characters (numbers of anal fin rays, dorsal fin rays, and vertebrae), earlier reported to differ 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 synonymized *S. 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. 1987*a*, 1987*b*) have demonstrated, however, 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 hybridization was reported between *S. aegyptiaca* and *S. senegalensis* Kaup 1858 (She et al. 1987*a*).

Tinti and Piccinetti (2000) examined nucleotide variation at two mitochondrial DNA (mtDNA) loci (*16S rRNA*, *cytochrome b*) in *Solea* spp. samples from the Mediterranean, the aim being "to provide an independent insight into the systematics of molecular characters which, with respect to the morphological ones, are free from subjective

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interpretations and environmental pressure". Surprisingly, the mtDNA sequences of their "*S. aegyptiaca*" sample appeared to be very close to the sequences of their *S. solea* sample. Those 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" that they eventually referred to as "*S. senegalensis*", with 39–41 vertebrae, and whose mtDNA 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 mtDNA 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 Atlantic–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 testing of the validity of a distinct genus *Pegusa* grouping *S. impar*, *S. lascaris*, and *S. nasuta* versus 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. (1987*a*, 1987*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 (*Aat-2*, *Gpi-1*, *Gpi-2*, *Ldh-2*, *Pt-3*) scored in common in all studies, and in five Atlantic–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 *S. aegyptiaca* from Zarzis, Tunisia (33°28′N, 11°07′E), sampled in May 2000, 8 *S. solea* from Pertuis Breton, France (46°19′N, 01°24′W), sampled in November 1999, 2 *S. solea* from an unknown location on the Atlantic coast of France sampled in June 2000, 4 *S. lascaris* from the Loire estuary, France (47°06′N, 02°20′W), sampled in June 2000, and 7 *S. lascaris* from Pertuis Charentais, France (45°48′N, 01°14′W), sampled in 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 *S. solea* (81.1 ± 1.7; N = 10) were in accordance with previous reports (Quignard et al. 1984, 1986). The DNA of each individual was extracted using phenol – chloroform – isoamyl alcohol, and a 354 base pair (bp) 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-denatured 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, Ohio, U.S.A.) with ^{33}P-labelled dideoxynucleotides (Amersham). All five sequences, which were deposited in GenBank (accession Nos. AF289716–AF289720), 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 two-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 bootstrap 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. *Microchirus variegatus* (Donovan, 1808) in the Soleidae was chosen as outgroup because its genetic distance from any *Solea* species is 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, personal observation).

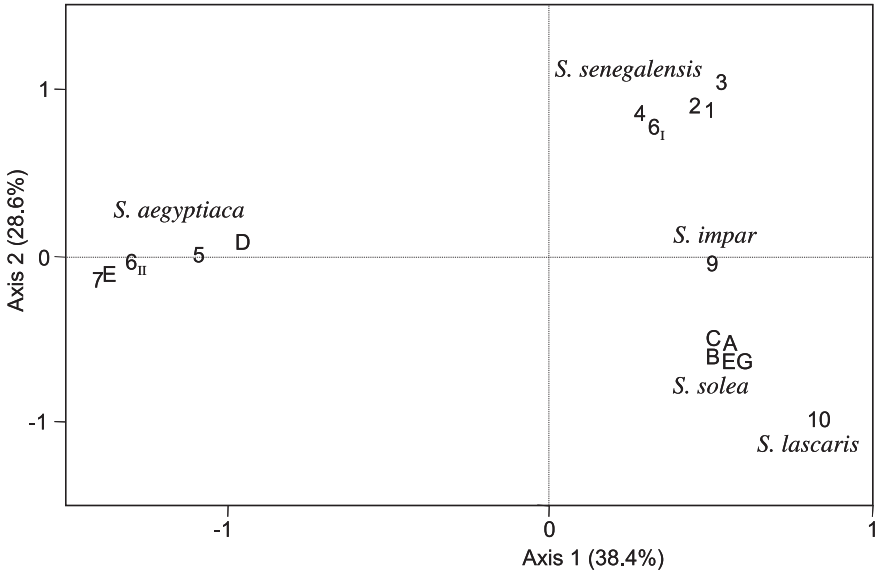
The systematic positions of *S. kleini* (Bonaparte, 1833) and *S. nasuta* are not addressed here because no sample material and no allozyme data for these species were available to us. However, partial nucleotide sequences of the cytochrome *b* gene and 16S rDNA of *S. kleini* have been presented by 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 MNHN 2000–5637 was designated as neotype, in conformity with the recommendations of the International Commission on Zoological Nomenclature (1999). The designation of a historical specimen collected by P. Chabanaud and preserved at MNHN as neotype for *S. aegyptiaca* is pending (J.-C. Hureau, personal communication in a letter).

Results and discussion

Although the distinction of *S. 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 that 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 to samples collected in the northeastern Atlantic, where the predominant species is *S. lascaris* (Marinaro 1988; J.-P. Quignard, personal observation). The distinction be-

Fig. 1. Correspondence analysis (BIOMECO package; Lebreton et al. 1990), showing projection on the plane defined by axis 1 and axis 2 (with percentages of total inertia in parenthese) of 16 Atlanto-Mediterranean *Solea* spp. samples. All samples (A, Brittany; B, C, D, E, 5, and 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 and 6_{II}, Gulf of Tunis, Tunisia; *7*, Khalîj-Qâbis, eastern Mediterranean; 10, Brittany) were characterized 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 are from Quignard et al. (1984); 1–5, 6_I, 6_{II}, and 7, from She et al. (1987*a*); 9 and 10 from Goucha et al. (1987); and EG from Kotoulas et al. (1995).



tween *S. impar* and *S. lascaris* was also evident from the distributions of the numbers of dorsal-fin and anal-fin rays compared with the values given for either of the type specimens examined by Ben Tuvia (Table VII in Ben Tuvia 1990). Numbers of vertebrae plotted against numbers of either anal-fin or dorsal-fin rays for individual "*Pegusa lascaris*" from the Atlantic and Mediterranean (including *S. impar* and *S. lascaris*, then considered synonyms; see Figs. 35 and 36 in 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 from the others. *Solea 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) are clustered onto a small spot in 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. 1997*b*). Such low levels of genetic heterogeneity across vast distances in each of these three taxa, and their clear separation from one another (Fig. 1), warrant their recognition as separate species, in spite of hybridization in areas of contact (between *S. aegyptiaca* and *S. senegalensis*; She et al. 1987*a*).

The most common mtDNA haplotype (cytochrome *b* gene) found by us in Atlantic *S. solea* (GenBank AF289716; frequency = 0.90) was identical with 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 with 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* hybridizes with *S. aegyptiaca* in a narrow zone of contact in northern Tunisia (She et al. 1987*a*), one cannot exclude the possibility that *S. senegalensis* might be introgressed by *S. aegyptiaca* mtDNA. 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 with that of the neighbor-joining tree and was supported by high bootstrap scores (Fig. 2). The phylogenetic relationships of *S. aegyptiaca*,

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 Atlantic–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-joining bootstrap values above branches, parsimony values below branches). Unlabelled nodes had bootstrap scores < 74%. Scale bar = 1% nucleotide divergence.

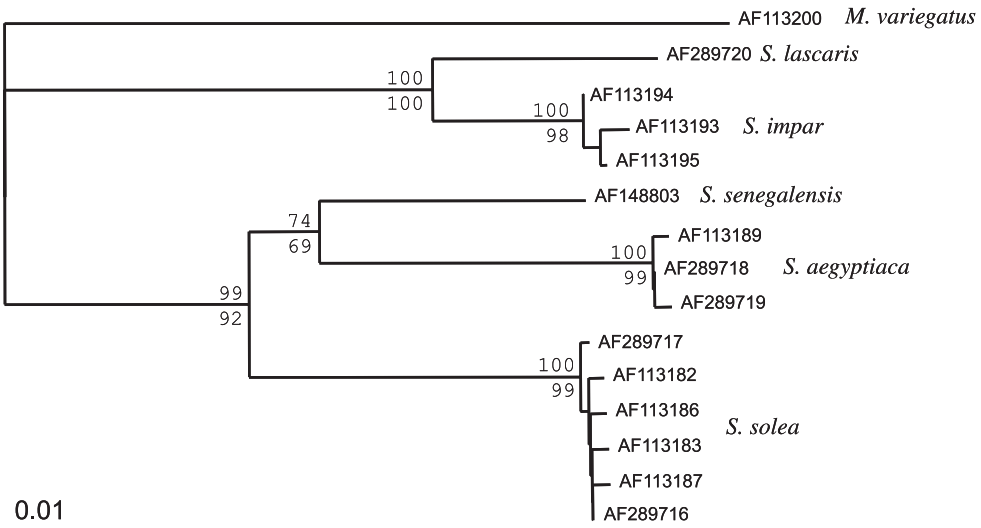
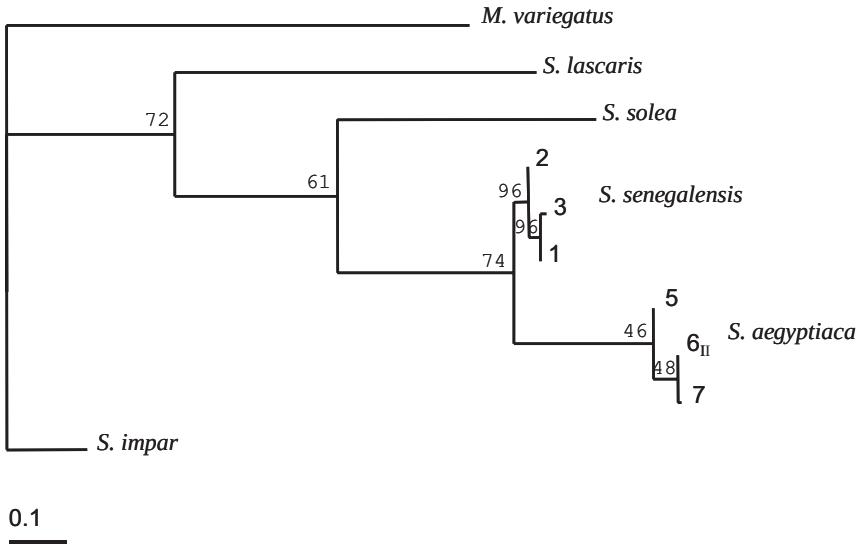


Fig. 3. Neighbor-joining tree (NEIGHBOR procedure in the PHYLIP package; Felsenstein 1993) derived from the matrix of pairwise Nei's (1972) genetic distances among 5 Atlantic–Mediterranean *Solea* species. Nei's distances were calculated using the GENDIST procedure of PHYLIP, from electromorph-frequency 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 five species and in outgroup *M. variegatus* by Goucha et al. (1987). Numbers at a node are percentages of occurrence after bootstrap resampling of loci (1000 bootstraps) using the procedure SEQBOOT of PHYLIP. Scale bar = 0.1 Nei's genetic distance.



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 providing no support for the ad hoc hypothesis that *S. senegalensis* possesses *S. aegyptiaca* mitochondria.

Thus, the distinction between *S. solea* and *S. aegyptiaca* fulfills the definitions of morphological species (Quignard et al. 1984; Ben Tuvia 1990), phylogenetic species (Goucha et al. 1987; present results), and biological species, since these two taxa are reproductively isolated throughout their range (Quignard et al. 1984; She et al. 1987*b*; this paper); the data of She et al. (1987*b*) also conform to the genotypic-cluster definition of species (Mallet 1995). The distinction between *S. impar* and *S. lascaris* likewise fulfills the definitions of morphological and phylogenetic species (Goucha et al. 1987; Ben Tuvia 1990; this paper). The congruence of phylogenetic relationships derived from such independent datasets as allozymes and cytochrome *b* gene sequences is both a powerful and a 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*, mtDNA or allozymes are equally reliable as characters for use in 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 a single character is used.

Chabanaud (1927) has suggested that *S. impar* and *S. lascaris* be grouped into the genus *Pegusa* Günther, 1862 s.str., on the basis of shared morphological features that are absent in the other Atlantic–Mediterranean *Solea* species (except *S. nasuta*), such as the anterior nostril on the blind side enlarged, rosette-shaped, and close to the posterior nostril. Bini (1968) and Desoutter (1990) again include *S. impar*, *S. lascaris*, and *S. nasuta* in the genus *Pegusa*. The mitochondrial tree (Fig. 2) lends some support to this distinction by grouping *S. impar* with *S. lascaris* as a separate clade, at the same time suggesting that the anterior nostril's shape and position are characters of phylogenetic value. As shown in the following points, the case for considering these to be 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 *M. variegatus* (frequency data in Goucha et al. 1987) had a steplike topology (Fig. 3). This tree did not exhibit separate clades for *S. aegyptiaca*, *S. senegalensis*, and *S. solea* versus *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 1.02–1.60). 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. (0.52–1.23; Bolch et al. 1994), but interspecific genetic-distance estimates within a genus generally prove lower, e.g., in mullets *Liza* spp. (0.29–0.48; Autem and Bonhomme 1980), tunas, *Thunnus* spp. (0.08–0.24; Elliott and Ward 1995), oreos, *Neocyttus* spp. (0.10–0.12; Lowry et al. 1996), flounders, *Platichthys* spp. (0.16–0.32; Borsa et al. 1997*a*), poor cods and bib, *Trisopterus* spp. (0.63–0.82; Mattiangeli 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 4.8–8.5%; Hoarau and Borsa 2000), *Centroberyx* spp. (range 6.9–12.3%; sequences in Hoarau 1999), *Decapterus* spp. (range 10.1–17.2%; Perrin and Borsa 2001), but more of the same order as distance estimates between closely related genera, e.g., *Beryx* spp. versus *Centroberyx* spp. (range 9.0–15.7%; sequences in Hoarau 1999) or *Decapterus* spp. versus *Selar crumenophthalmus* (range 19.1–26.2%; Perrin and Borsa 2001). In our view this is a sufficient argument in support of a distinct genus, namely *Pegusa*.

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