Genetics and Molecular Biology, 31, 1 (suppl), 284-292 (2008)

www.sbg.org.br

Research Article

Phylogenetic analysis of the order Pleuronectiformes (Teleostei) based on sequences of 12S and 16S mitochondrial genes

Marisa F.C. Azevedo[1*], Claudio Oliveira[1], Belén G. Pardo[2], Paulino Martínez[2] and Fausto Foresti[1]

[1]*Laboratório de Biologia e Genética de Peixes, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, SP, Brazil.*
[2]*Laboratorio de Genética, Faculdade de Veterinaria, Universidad de Santiago de Compostela, Lugo, Spain.*

Abstract

The fish order Pleuronectiformes, composed of 14 families, has two suborders: Psettodoidei (with one family) and Pleuronectoidei (with thirteen families). The relationships among families of Pleuronectoidei and among the genera of their families have extensively been debated and a consensus has not yet been reached. In the present study, partial sequences of the 12S and 16S mitochondrial rRNA genes were obtained from 19 species belonging to the families Achiridae, Bothidae, Cynoglossidae, Paralichthyidae, Pleuronectidae, Scophthalmidae, and Soleidae. Additional sequences of 42 pleuronectiform species were obtained from GenBank. Phylogenetic analyses were conducted by the methods of maximum-parsimony, maximum-likelihood and Bayesian inference. Our results corroborate the monophyletic status of all families, excluding Paralichthyidae. In the family Achiridae, the genus *Catathyridium* (freshwater) was the sister group of *Trinectes* (saltwater), and *Hypoclinemus* (freshwater) was the sister group of *Achirus* (saltwater). Assuming that the putative ancestor of achirids lived in saltwater, it is suggested that the freshwater habitats in South America were colonized independently by different achirid lineages.

Key words: phylogeny, molecular systematics, mitochondrial DNA, fish evolution, flatfishes.

Received: September 11, 2006; Accepted: May 14, 2007.

Introduction

Flatfishes of the Pleuronectiformes order are easily recognized because the adults are not bilaterally symmetrical (Nelson, 2006). Young flatfishes are bilaterally symmetrical and swim upright, but early in their development one eye migrates across the top of the skull to lie adjacent to the eye on the other side (Ahlstrom *et al.*, 1984). Most species have both eyes on the right side and lie on the left side (dextral) but some suffer an opposite development (sinistral) (Nelson, 2006). In some species, such as *Platichthys stellatus*, both dextral and sinistral individuals may occur and this difference appears to be largely under genetic control (Policansky, 1982). However, there appears to be no convincing arguments for a direct adaptive advantage for being sinistral or dextral (Nelson, 2006). Many flatfish species have great economical importance, mainly due to the quality of their meat, and have been extensively exploited (Cerqueira *et al.*, 1997).

Send correspondence to Marisa Fagundes Carvalho de Azevedo. Núcleo em Ecologia e Desenvolvimento Sócio-Ambiental de Macaé, Universidade Federal do Rio de Janeiro, NUPEM/UFRJ, Caixa Postal 119331, 27910-970 Macaé, RJ, Brazil. E-mail: marisafca@yahoo.com.
*Present address: Núcleo em Ecologia e Desenvolvimento Sócio-Ambiental de Macaé, Universidade Federal do Rio de Janeiro, Macaé, RJ, Brazil.

Regan (1910, cited by Ramos, 1998) proposed the first morphological classification for the order then named Heterostomata. The author considered the group as monophyletic and divided it in two putative monophyletic suborders, Psettodoidei and Pleuronectoidei. Currently, the main classification of the order is still based on morphological characters. However, the monophyletic nature of several families has been extensively debated (Chapleau, 1993; Chapleau and Keast, 1988; Chanet, 1997; Fukui, 1997; Hensley, 1997; Cooper and Chapleau, 1998; Berendzen and Dimmick, 2002; Pardo *et al.*, 2005).

The most recent and extensive phylogenetic study of Pleuronectiformes using morphological data was done by Chapleau (1993). According to this author, Psettodidae is the most primitive family, while Cynoglossidae and Soleidae are the most derived. Citharidae, Scophthalmidae, Paralichthyidae, Bothidae, Pleuronectidae, and Achiridae are considered intermediary families. According to the classification proposed by Nelson (2006), mainly based on the studies of Chapleau (1993), Cooper and Chapleau (1998) and Hoshino (2001), the order has 14 families. Psettodidae is the only family of the suborder Psettodoidei and the remaining families belong to the suborder Pleuronectoidei.

The first molecular study conducted on Pleuronectiformes by Tinti *et al.* (1999) reinforced the previously proposed relationships based on morphological characters, but only nine species were investigated. Pardo *et al.* (2005), analyzed fragments of about 650 base pairs (bp) of the 16S mtDNA gene of 30 species, and Berendzen and Dimmick (2002) analyzed DNA sequences of about 1200 bp of the 12S and 16S mtDNA genes of 44 species. These analyses resulted in well-resolved phylogenies at species and genus levels. However, the relationship among families remained unresolved.

Most species of flatfishes live in saltwater or brackish water, but some are also found in freshwater (Nelson, 2006). Among the few freshwater species those of the family Achiridae are encountered in Brazil. Achirids are mainly shore fishes restricted to both sides of Americas (Ramos, 2003a). According to Ramos (1998), the most primitive achirid species are freshwater species of the genera *Hypoclinemus* and *Catathyridium*. In the present study, partial sequences of the mitochondrial genes 12S and 16S of mtDNA of 19 species belonging to seven families of Pleuronectiformes were determined with the aim of investigating the monophyletic nature of the recognized families, mainly that of the South America achirids.

Materials and Methods

Tissue samples

Tissue samples were obtained from specimens of 19 species of Pleuronectiformes belonging to the families Achiridae, Bothidae, Cynoglossidae, Paralichthyidae, Pleuronectidae, Scophthalmidae, and Soleidae. Species names and sampling sites are shown in Table 1. Additional sequences of 42 pleuronectiform species were retrieved from GenBank (Table 1). The single species of Psettodidae (*Psettodes erumei*) was used as outgroup, following the results by Chapleau (1993) who demonstrated that this is the most primitive family among Pleuronectiformes.

Table 1 - Specimens analyzed in the present study and respective GenBank access numbers. FW = freshwater; BW = brackish water; SW = salt water. Asterisks show the species sequenced in the present study.

Family	Species	Source	12S rRNA	16S rRNA
Achiridae	*Achirus declivis**	BW, Pará, Brazil	*AY998041*	*AY998029*
	*Catathyridium jenynsi**	FW, Paraná, Brazil	*AY998034*	*AY998022*
	*Hypoclinemus mentalis**	FW, Acre, Brazil	*AY998035*	*AY998023*
	*Trinectes paulistanus**	SW, São Paulo, Brazil	*AY998036*	*AY998024*
	Trinectes maculatus	GenBank	*AF488496*	*AF488446*
Bothidae	*Arnoglossus imperialis**	SW, Spain	*AY141358*	*AY359651*
	Arnoglossus thori	GenBank	*AF542208*	*AY157329*
	Arnoglossus laterna	GenBank	*AF542210*	*AY359653*
	Bothus lunatus	GenBank	*AF488508*	*AF488458*
	*Bothus ocellatus**	SW, São Paulo, Brazil	*AY998038*	*AY998026*
	Bothus podas	GenBank	*AF542221*	*AY157326*
	Bothus robinsi	GenBank	*AF488509*	*AF488459*
	Crossorhombus kobensis	GenBank	*AF488506*	*AF488456*
	Laeops kitaharae	GenBank	*AF488511*	*AF488461*
Citharidae	*Citharoides macrolepis*	GenBank	*AF488513*	*AF488463*
	Citharus linguatula	GenBank	*AF542220*	*AY157325*
Cynoglossidae	*Symphurus plagusia**	SW, São Paulo, Brazil	*AF488497*	*DQ017374*
	*Symphurus tesselatus**	SW, São Paulo, Brazil	*AY998037*	*AY998025*
Paralichthyidae	*Citharichthys xanthostigma*	GenBank	*AF488499*	*AF488449*
	*Cyclopsetta chittenden*i*	SW, São Paulo, Brazil	*AY998031*	*AY998019*
	*Etropus crossotus**	SW, São Paulo, Brazil	*AY998032*	*AY998020*
	*Etropus longimanus**	SW, São Paulo, Brazil	*AY998033*	*AY998021*
	Etropus microstomus	GenBank	*AF488502*	*AF488452*
	Paralichthys dentatus	GenBank	*AF488501*	*AF488451*
	*Paralichthys patagonicus**	SW, Santa Catarina, Brazil	*AY998040*	*AY998028*
	Pseudorhombus pentophthalmus	GenBank	*AF488505*	*AF488455*
	*Syacium papillosum**	SW, São Paulo, Brazil	*AY998039*	*AY998027*
	Tarphops oligolepis	GenBank	*AF488507*	*AF488457*
	Xystreurys liolepis	GenBank	*AF488504*	*AF488454*

Table 1 (cont.)

Family	Species	Source	12S rRNA	16S rRNA
Pleuronectidae	*Eopsetta jordani*	GenBank	*AF488476*	*AF488426*
	Glyptocephalus zachirus	GenBank	*AF488486*	*AF488436*
	Hippoglossus stenolepis	GenBank	*AF488483*	*AF488433*
	Isopsetta isolepis	GenBank	*AF488481*	*AF488431*
	Lepidopsetta bilineata	GenBank	*AF488479*	*AF488429*
	Limanda aspera	GenBank	*AF488491*	*AF488441*
	Lyopsetta exilis	GenBank	*AF488484*	*AF488434*
	Microstomus bathybius	GenBank	*AF488490*	*AF488440*
	Microstomus pacificus	GenBank	*AF488480*	*AF488430*
	Parophrys vetula	GenBank	*AF488488*	*AF488438*
	*Platichthys flesus**	SW, Spain	*AF542206*	*AY359670*
	Platichthys stellatus	GenBank	*AF488482*	*AF488432*
	Pleuronectes platessa	GenBank	*AF542207*	*AY157328*
	Pleuronichthys guttulatus	GenBank	*AF488487*	*AF488437*
	Pleuronichthys verticalis	GenBank	*AF488489*	*AF488439*
	Psettichthys melanostictus	GenBank	*AF488485*	*AF488435*
	Pseudopleuronectes americanus	GenBank	*AF488478*	*AF488428*
Psettodidae	*Psettodes erumei*	GenBank	*AF488518*	*AF488468*
Samaridae	*Samariscus xenicus*	GenBank	*AF488517*	*AF488467*
	Plagiopsetta glossa	GenBank	*AF488516*	*AF488466*
Scophthalmidae	*Lepidorhombus wiffiagonis**	SW, Spain	*AY998042*	*AY998030*
	Scophthalmus aquosus	GenBank	*AF488512*	*AF420449*
	*Scophthalmus maximus**	SW, Spain	*AY998043*	*AY998019*
	*Scophthalmus rhombus**	SW, Spain	*AY998044*	*AY998020*
Soleidae	*Aseraggodes kobensis*	GenBank	*AF488493*	*AF488443*
	*Dicologlossa cuneata**	SW, Spain	*AF542211*	*AY359660*
	Heteromycteris japonicus	GenBank	*AF488494*	*AF488444*
	Microchirus azevia	GenBank	*AF542216*	*AY157318*
	Microchirus ocellatus	GenBank	*AF542218*	*AF112850*
	Microchirus variegatus	GenBank	*AY141359*	*AF112851*
	Monochirus hispidus	GenBank	*AF542219*	*AF112852*
	Solea solea	GenBank	*AF488492*	*AF112845*
	*Solea sonegalensis**	SW, Spain	*AF542205*	*AY359661*

DNA extraction and sequencing

Total DNA was extracted from ethanol-preserved liver or muscle tissue with the Wizard Genomic DNA Purification Kit (Promega). Partial sequences of the mitochondrial genes 12S and 16S rRNA were amplified by the polymerase chain reaction (PCR) with the following primers: L1091 and H1478 (Kocher *et al.*, 1989) for the gene 12S rRNA, and 16Sa-L and 16Sb-H (Palumbi *et al.*, 1991) for the gene 16S rRNA. Primer concentrations were 5 µM. Amplifications were performed in a total volume of 25 µL for 35 cycles (30 s at 95 °C, 60 s at 50-60 °C, and 120 s at 72 °C). The PCR products were visualized on a 1% agarose gel. The amplified segments were extracted from the gel with the kit GFX PCR DNA and Gel Purification (GE Healthcare). Sequencing reactions were performed with the kit Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction (Applied Biosystems) and analyzed in an automated sequencer ABI Prism 377 DNA Sequencer (Applied Biosystems). Some sequencing reactions were done with the kit Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) and analyzed in an automated sequencer model ALF Express II (Amersham Biosciences). All sequences were read at least two times (forward and reverse).

Sequence analyses and phylogenetic approaches

Individual sequences of each species were initially analyzed with the software DAMBE (Xia and Xie, 2001) and a consensus sequence was obtained for each DNA segment of each species. After that, all sequences were aligned

with the software Clustal W, implemented in DAMBE (Xia and Xie, 2001). Nucleotide variation, substitution patterns and genetic distances were examined using MEGA 2.1 (Kumar *et al.*, 2001). Nucleotide saturation was analyzed by plotting the absolute number of transitions (Ti) and transversions (Tv) against genetic distance values in the software DAMBE (Xia and Xie, 2001).

Maximum-parsimony (MP) based phylogenetic analyses were performed using the software PAUP* beta version 4.0b10 (Swofford, 2002) with heuristic searches using random addition of sequences and the tree bisection and reconnection (TBR) algorithm. In all analyses, the character-state optimization method employed was the accelerated transformation (ACCTRAN). Parsimony trees were generated using Ti/Tv ratios of 1:1 and 1:2, considering gaps as missing data or as a fifth base. Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 1000 replicates with random additions and TBR branch swapping.

Genetic distances among sequences were estimated by the Hasegawa-Kishino-Yano (HKY85) nucleotide substitution model (Hasegawa *et al.*, 1985), incorporating the proportion of invariant sites and among-site rate heterogeneity based on a hierarchical hypothesis test of alternative models implemented in the program Modeltest 3.7 (Posada and Crandall, 1998). The Bayesian-inference method of phylogenetic analysis (Huelsenbeck *et al.*, 2001) was used to evaluate alternative tree topologies through the estimation of posterior probabilities using MrBayes v. 3.0 (Ronquist and Huelsenbeck, 2003). The MrBayes analysis ran four chains simultaneously, each for 1 million generations. Every 100th generation was sampled and the asymptote of the likelihood score was detected with the SUMP command. All sampled topologies beneath the asymptote were discarded from the population of trees considered in the subsequent majority-rule consensus. The frequency with which a particular clade appeared in the population of retained topologies was interpreted as its posterior probability. Posterior probabilities were interpreted as a measure of how likely the clade appears in the optimal topology, rather than accuracy of the node with respect to species relationships or clade stability. Consensus trees were produced with the software TreeExplorer implemented in MEGA 2.1 (Kumar *et al.*, 2001).

Maximum likelihood (ML) phylogenetic analyses were performed using the software PHYML (Guindon and Gascuel, 2003) in its website version (Guindon *et al.*, 2005) using the Hasegawa-Kishino-Yano (HKY85) nucleotide substitution model (Hasegawa *et al.*, 1985). Clade stability was estimated by non-parametric bootstrapping in 500 replicates with PHYML.

Results

Sequences obtained for this study have been deposited in GenBank (accession numbers AY998019 - AY998044) (Table 1).

The combined sequence data of the 61 flatfish taxa resulted in 1428 bp, of which 314 were conserved sites and 981 were parsimony informative. The transition/transversion (Ti/Tv) ratio observed was 0.9. Percent base composition for sequenced regions of the L-strand was determined as follows: adenine (A) 29.4; cytosine (C) 26.1; guanine (G) 22.3; and thymine (T) 22.2. The analysis of these data clearly shows that the base composition of the L-strand is somewhat A-rich, similar to that described for several mitochondrial genes of fishes (Alves-Gomes *et al.*, 1995; Shimabukuro-Dias *et al.*, 2004), lizards (Reeder, 1995), and snakes (Parkinson, 1999). The results from the analysis of plotting transitions and transversions against genetic distance suggest the occurrence of low nucleotide saturation (Figure 1).

Initially, a total of eight MP heuristic searches were conducted employing all data or excluding 179 characters belonging to six segments with alignment problems, including or excluding gaps, and considering the Ti/Tv ratios of 1:1 or 1:2. The resultant phylogenies were largely congruent. The best resolved MP consensus tree obtained from the analysis of 1000 bootstrap replicates including all positions, treating gaps as missing data, and the Ti/Tv ratio of 1:1 is presented in Figure 2. For this tree, the results obtained were: tree length = 4332, consistency index (CI) = 0.4084, homoplasy index (HI) = 0.5916 and retention index (RI) = 0.6259.

Phylogenetic analyses with the Bayesian method resulted in a similar tree with the nodes supported by values usually higher than those found in MP analysis (Figure 3). The phylogeny obtained with the ML method was similar to that obtained with the MP method (Figure 4) but some families were differently positioned.

Discussion

Considering Psettodidae as sister group of all other pleuronectiforms (Chapleau, 1993), all remaining families investigated in our study appeared as monophyletic (with the exception of Paralichthyidae) and were supported by

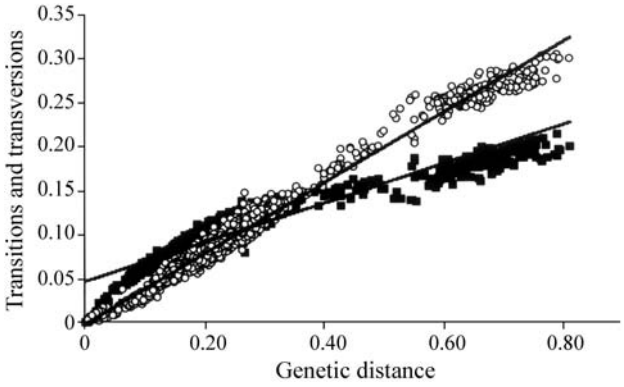

Figure 1 - Graphic showing the frequency of observed transitions and transversions versus genetic distance (Kimura, 1980) of 12S and 16S rRNA genes. Transitions are black squares, transversions are open circles.

high bootstrap and posterior probability values. However, the data obtained did not resolve the relationship among several families, as already observed in previous studies employing 12S and 16S rRNA sequences (Berendzen and Dimmick, 2002; Pardo *et al.*, 2005).

Paralichthyidae with about 16 genera and 105 species (Nelson, 2006), has been recognized as a paraphyletic group (Hensley and Ahlstrom, 1984; Chapleau, 1993; Pardo *et al.*, 2005; Berendzen and Dimmick, 2002). In the present study, the analysis of specimens from eight genera showed that they belong to two or three independent lineages. The group identified as Paralichthyidae 1 was related to Bothidae in all analyses (Figures 2 to 4). The group identified as Paralichthyidae 2 in the MP and ML analyses appeared as polyphyletic (Figures 2 and 4), as also observed in the Bayesian analysis (Figure 3). The first group (Paralichthyidae 1), composed by the genera *Cyclopsetta*, *Syacium*, *Citharichthys*, and *Etropus* was previously recognized by Chapleau (1993) as a natural group (named *Cyclopsetta* group) sharing a urinary papilla oriented towards the blind size, an ocular pelvic fin based on the mid-ventral line of the body, a blind size pelvic-fin base an-

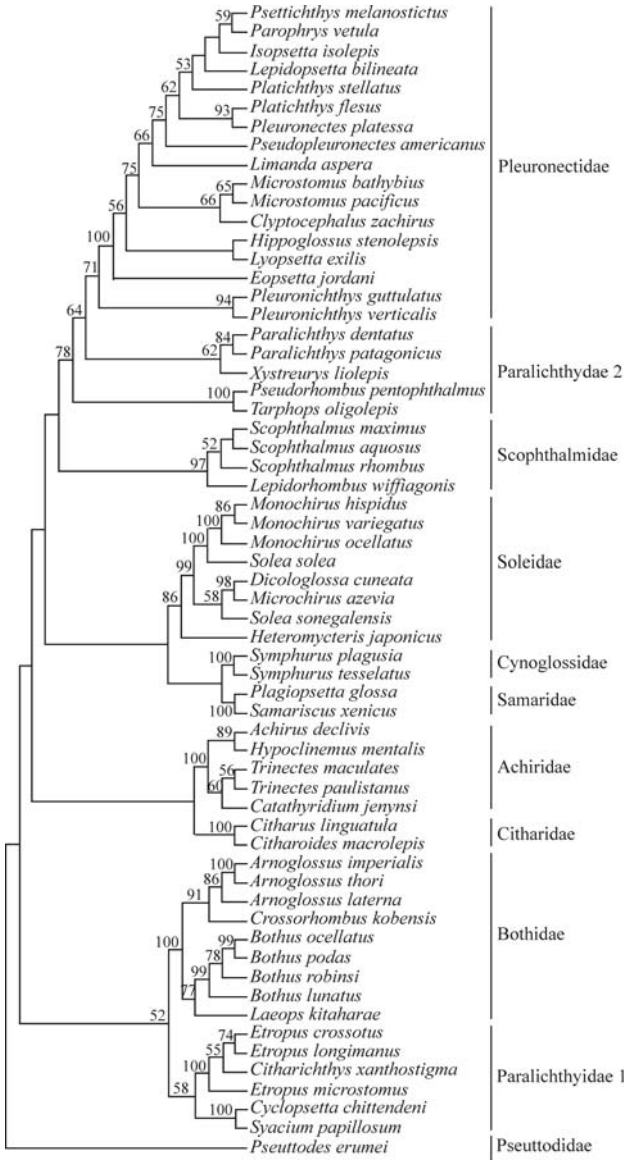

Figure 2 - Pleuronectiforms consensus maximum-parsimony tree produced when gaps were treated as missing data and the Ti/Tv ratio of 1:1 (TL = 4332, CI = 0.4084, HI = 0.5916, RI = 0.6259). Numbers above branches are bootstrap values based on 1000 replicates. Values below 50% are not shown.

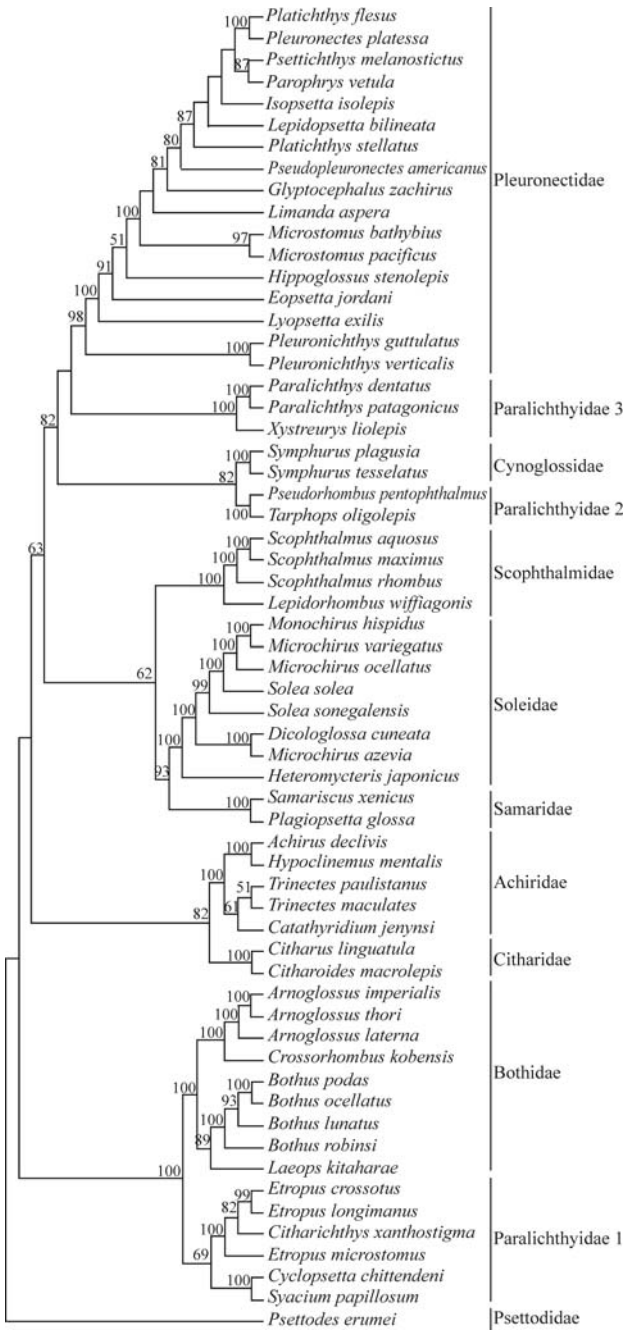

Figure 3 - Pleuronectiformes consensus tree produced by a Bayesian analysis. Numbers above nodes are posterior probabilities recovered by the Bayesian analysis. Values below 50% are not shown.

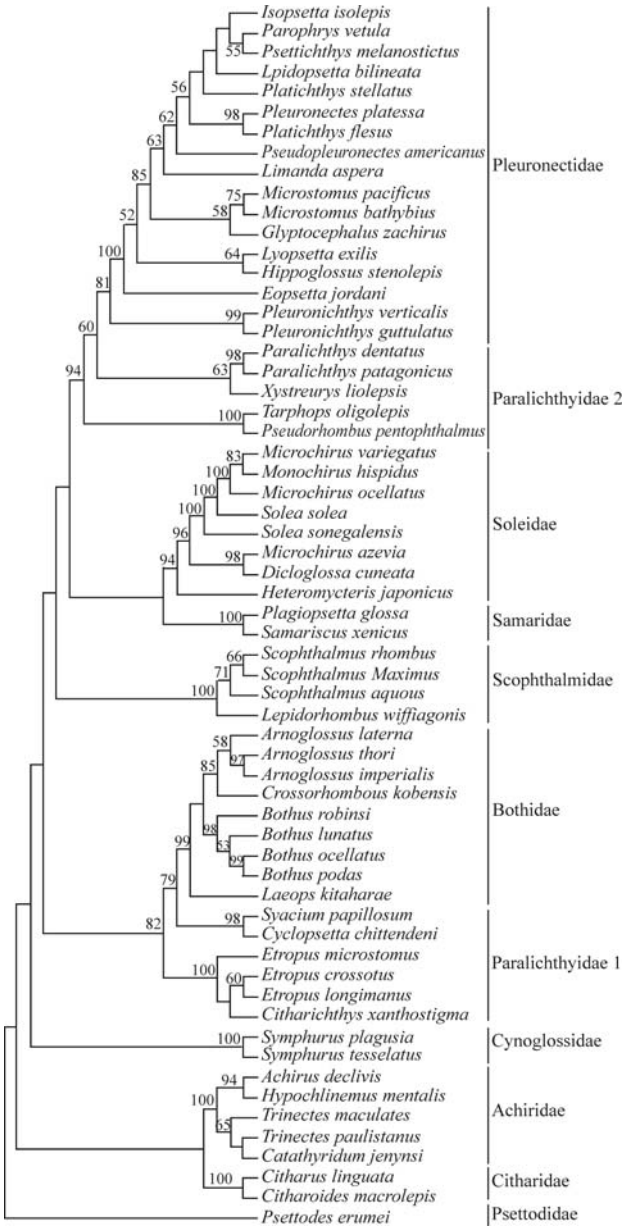

Figure 4 - Pleuronectiformes consensus tree produced by a maximum-likelihood analysis (-ln = 20707.22). Numbers above branches are bootstrap values based on 1000 replicates. Values below 50% are not shown.

terior to that of the ocular side, a caudal fin with 17 rays, none of which are supported by preural, neural or hemal spines, and hypural 5 fused with the epural. A molecular relationship among *Syacium*, *Citharichthys*, and *Etropus* was also established by Berendzen and Dimmick (2002).

Hensley and Ahlstrom (1984) indicated a possible close relationship for the *Cyclopsetta* group with the bothids on the basis of the absence of a first neural spine and the presence of vertebral apophyses. A possible relationship between the bothids and the *Cyclopsetta* group was also pointed out in the present study and in other molecular studies (Berendzen and Dimmick, 2002; Pardo *et al.*, 2005). The genus *Etropus* was found to be paraphyletic, with the species *E. microstomus* related to *Citharichthys xanthostigma*. Interestingly, the species *E. microstomus* is distributed from New Jersey to North Carolina and *C. xanthostigma* is found in Magdalena Bay, Baja California, Mexico, while the species *E. crossotus* and *E. longimanus* are found across the Atlantic Ocean coasts of South America (Eschmeyer, 1998).

The Paralichthyidae 2 group appeared here as a paraphyletic group composed of two units (independently identified in the Bayesian analysis). One of these lineages with a 100% support included the genera *Pseudorhombus* and *Tarphops* as observed also by Berendzen and Dimmick (2002). These two genera plus the genus *Cephalopsetta* were recognized by Chapleau (1993) as the *Pseudorhombus* group, a possible monophyletic lineage among paralichthyids. The third monophyletic lineage found in the Paralichthyidae was composed of the genera *Paralichthys* and *Xystreurys*. Some morphological evidence of monophyly had been observed for these genera and the remained paralichthyids (Chapleau, 1993), but previous molecular studies (Berendzen and Dimmick, 2002) also corroborated the hypothesis that these groups belong to a monophyletic clade.

The families Citharidae, Cynoglossidae, Samaridae, and Scophthalmidae were represented by a reduced number of species in the present study, in spite of this, they were found to be monophyletic in all analyses. The family Citharidae with five genera and six species (Nelson, 2006) was represented in this study by two genera, *Citharus* and *Citharoides.* These two genera comprise sinistral species and appeared as monophyletic in all analyses realized. However, this family was described previously as a polyphyletic group (Hensley and Ahlstrom, 1984; Chapleau, 1993; Hoshino and Amaoka, 1998; Berendzen and Dimmick, 2002).

The family Cynoglossidae comprises three genera and 127 species (Nelson, 2006). In the present study, the two species of the genus *Symphurus* appeared as a monophyletic group. The monophyly of Cynoglossidae had already been proposed by several authors (Hensley and Ahlstrom, 1984; Chapleau, 1993; Hoshino and Amaoka, 1998; Berendzen and Dimmick, 2002; Pardo *et al.*, 2005).

The family Samaridae comprises three genera and about 20 species (Nelson, 2006). The monophyly of Samarinae was proposed by Sakamoto (1984) and confirmed by Chapleau (1993). These results permitted to raise the subfamily Samarinae to the family level by Nelson (2006). In the present study, the two genera analyzed, *Plagiopsetta* and *Samariscus*, appeared as belonging to a monophyletic group. Previous molecular studies also corroborated the monophyly of this family (Berendzen and Dimmick, 2002; Pardo *et al.*, 2005).

The family Scophthalmidae comprises four genera and about eight species (Nelson, 2006). In the present study, four species belonging to two genera were analyzed.

The monophyly of Scophthalmidae as well as of the genus *Scophthalmus* was observed in all conducted analyses. This family has been recognized as monophyletic by several authors (Hensley and Ahlstrom, 1984; Chapleau, 1993; Hoshino and Amaoka, 1998; Berendzen and Dimmick, 2002; Pardo *et al.*, 2005).

The family Soleidae is represented here by six of its 35 genera (Nelson, 2006). This family was found to be monophyletic with a high posterior probability in the Bayesian analysis. The monophyly of Soleidae previously proposed by Desoutter and Chapleau (1997) was corroborated by Pardo *et al.* (2005), although controversial data was reported by Berendzen and Dimmick (2002). Although the relationships among the genera were well supported, the species phylogeny suggested that the genera *Solea* and *Microchirus* are not monophyletic.

The family Bothidae has 20 genera and about 140 species (Nelson, 2006). The four genera analyzed in the present study appeared as a monophyletic group. The monophyletic nature of Bothidae was proposed by Hensley and Ahlstrom (1984) and Chapleau (1993) and corroborated in an extensive study conduced by Fukui (1997), where the author listed five synapormophies for the family.

The family Pleuronectidae is one of the largest families of flatfishes. This family was formerly split into the subfamilies Pleuronectinae, Poecilopsettinae, Rhombosoleinae and Samarinae but phylogenetic studies reviewed by Chapleau (1993) showed that these groups did not formed a monophyletic assemblage and should be ranked at family level. Thus, in a more recent phylogenetic study Cooper and Chapleau (1998) recognized that the name Pleuronectidae should only be employed for the species of the old subfamily Pleuronectinae, as followed here. Based on their revision, Cooper and Chapleau (1998) showed that the family Pleuronectidae is a monophyletic group defined by ten synapomorphies. The present results, based on 14 out of the 23 currently recognized genera of Pleuronectidae, confirm the monophyly of the group as suggested in previous molecular studies (Berendzen and Dimmick, 2002; Pardo *et al.*, 2005). However, the division of this family into subgroups should be reevaluated. The first division proposed by Li (1981) was completely refuted since members of its tribes Hippoglossini and Pleuronectini did not form monophyletic groups. The Cooper and Chapleau (1998) proposal was partially corroborated. Thus, the monophyly of the subfamily Eopsettinae, as well as of the tribe Isopsettini were corroborated, but the remaining divisions were not. Additional molecular data are necessary for a better understanding of the relationships among pleuronectids.

The family Achiridae has seven genera and about 33 species (Ramos, 2003b, Nelson, 2006). The monophyly of the family was proposed by Chapleau and Keast (1988), and Chapleau (1993), and corroborated in an extensive revision conducted by Ramos (1998). In the present study, the analysis of four genera showed that they belong to a monophyletic group. The genus *Catathyridium* (freshwater species) appeared as the sister group of *Trinectes* (marine species) and the genus *Hypoclinemus* (freshwater species) as the sister group of *Achirus* (marine species). This hypothesis is different from that presented by Ramos (1998). This author found that *Hypoclinemus* and *Catathyridium* were primitive sister groups to all other achirids and proposed that this family could have originated in freshwater. Based only on our data for the family Achiridae it is not possible to decide whether this family originated in marine or freshwater environments. However, considering that the sister groups of Achiridae are families found exclusively in saltwater, the most parsimonious hypothesis is that the achirid freshwater species of the genera *Hypoclinemus* and *Catathyridium* derived directly and independently from saltwater ancestors. The genetic divergence, estimated by the HKY85 model (Hasegawa *et al.*, 1985) between the genera *Catathyridium* and *Trinectes* was only 7.45% and between the genera *Hypoclinemus* and *Achirus* it was only 5.4%, supporting a recent origin of these genera. The achirid consensus MP tree (Figure 2) and the tree satisfying the alternative hypotheses that freshwater species were sister-groups were tested using the Wilcoxon signed-rank test (Templeton, 1983) and the maximum-likelihood ratio test (Kishino and Hasegawa, 1989) implemented in PAUP*. Both tests rejected the null hypothesis that the alternative phylogenies were not statistically different (Templeton test $z = -3.6556$, $p = 0.0003$; Kishino - Hasegawa test $t = 3.6826$, $p = 0.0002$) reinforcing the hypothesis that freshwater species originated from different marine ancestors.

The present analyses corroborated the monophyletic status of most families of flatfish: Scophthalmidae, Pleuronectidae, Samaridae, Cynoglossidae, Achiridae, Citharidae, and Bothidae, but excluding the family Paralichthyidae which was shown to be polyphyletic. The polyphyletic origin of freshwater species suggests independent colonization events of continental waters by members of the family Achiridae. Future analyses of additional species and mainly on larger genomic regions will be necessary for a better understanding of the relationships among flatfish groups, especially with respect to interfamilial relationships.

Acknowledgments

The authors are grateful to Renato Devidé for his technical assistance, to Robson T. C. Ramos and Guaracy Tadeu Rocha for a critical reading of the manuscript and to Naércio A. Menezes, José Lima Figueiredo and Robson T. C. Ramos for identifying specimens. Funds supporting this study were provided by CNPq, CAUNESP and FAPESP, and Universidad de Santiago de Compostela.

References

Ahlstrom EH, Amaoka K, Hensley DA, Moser HG and Sumida BY (1984) Pleuronectiformes development. In: Moser HG,

Richards WJ, Cohen DM, Fahay MP, Kendall AW and Richardson SL (eds) Ontogeny and Systematic of Fishes. Special Publication n. 1. American Society of Ichthyology and Herpertology, Lawrence, pp 640-670.

Alves-Gomes J, Ortí G, Haygood M, Heiligenberg W and Meyer A (1995) Phylogenetic analysis of the South American electric fishes (Order Gymnotiformes) and the evolution of their electrogenic system: A synthesis based on morphology, electrophysiology, and mitochondrial sequence data. Mol Biol Evol 12:298-318.

Berendzen PB and Dimmick WW (2002) Phylogenetic relationships of Pleuronectiformes based on molecular evidence. Copeia 2002:642-652.

Cerqueira VR, Mioso R, Macchiavello JAG and Berger AM (1997) Ensaios de indução de desova do linguado (*Paralichthys orbignyanus* Valenciennes, 1839). Bol Inst Pesca 24:247-254.

Chanet B (1997) A cladistic reappraisal of the fossil flatfish's record consequences on the phylogeny of the Pleuronectiformes (Osteichthyes, Teleostei). Ann Sci Nat 18:105-117.

Chapleau F (1993) Pleuronectiform relationships: A cladistic reassessment. Bull Mar Sci 52:516-540.

Chapleau F and Keast A (1988) A phylogenetic reassessment of the monophyletic status of the family Soleidae, with notes on the suborder Soleoidei. Can J Zool 66:2797-2810.

Cooper JA and Chapleau F (1998) Phylogenetic status of *Paralichthodes algoensis* (Pleuronectiformes, Paralichthyidae). Copeia 1998:477-481.

Desoutter M and Chapleau F (1997) Taxonomic status of *Bathysolea profundicola* and *B. polli* (Soleidae, Pleuronectiformes) with notes on the genus. Ichthyol Res 44:399-412.

Eschmeyer WN (1998) Catalog of Fishes. California Academy of Sciences, San Francisco, 2905 pp.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Fukui A (1997) Early ontogeny and systematics of Bothidae, Pleuronectoidei. Bull Mar Sci 60:192-212.

Guindon S and Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Sys Biol 52:696-704.

Guindon S, Lethiec F, Duroux P and Gascuel O (2005) PHYML Online - A web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res 33:557-559.

Hasegawa M, Kishino H and Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160-174.

Hensley DA (1997) An overview of the systematics and biogeography of the flatfishes. J Sea Res 37:187-194.

Hensley DA and Ahlstrom EH (1984) Pleuronectiformes: Relationships. Pleuronectiformes: Development. In: Moser HG, Richards WJ, Cohen DM, Fahay MP, Kendall AW and Richardson SL (eds) Ontogeny and Systematic of Fishes. Special Publication n. 1. American Society of Ichthyology and Herpertology, Lawrence, pp 670-687.

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.

Huelsenbeck JP, Ronquist F, Nielsen R and Bollback JP (2001) Bayesian inference of phylogeny and its impact of evolutionary biology. Science 294:2310-2314.

Kishino H and Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J Mol Evol 29:170-179.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX and Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86:6196-6200.

Kumar S, Tamura K, Jakobsen I and Nei M (2001) MEGA: Molecular evolutionary genetic analysis, v. 2.1. Pennsylvania State University, University Park, and Arizona State University, Tempe.

Li SZ (1981) On the origin, phylogeny and geographical distribution of the flatfishes (Pleuronectiformes). Trans Chin Ichthyol Soc 1981:11-20.

Nelson JS (2006) Fishes of the World. 4th ed. Wiley, New York, 601 pp.

Palumbi S, Martin A, Romano S, McMillan WO, Stice L and Grabowski G (1991) The Simple Fool's Guide do PCR, v. 2.0. University of Hawaii, Honolulu, 43 pp.

Pardo BG, Machordom A, Foresti F, Porto-Foresti F, Azevedo MFC, Bañon R, Sánchez L and Martinez P (2005) Phylogenetic analysis of flatfishes (order Pleuronectiformes) based on mitochondrial 16S rDNA sequences. Sci Mar 69:531-543.

Parkinson CL (1999) Molecular systematics and biogeographical history of Pitvipers as determined by mitochondrial ribosomal DNA sequences. Copeia 1999:576-586.

Policansky D (1982) The asymmetry of flounders. Sci Am 246:116-122.

Posada D and Crandall KA (1998) Modeltest: Testing the model of DNA substitution. Bioinformatics 14:817-818.

Ramos RTC (1998) Estudo filogenético da família Achiridae (Teleostei, Pleuronectiformes, Pleuronectoidei), com a revisão das formas de água doce da América do Sul Cis-Andina e a reavaliação do monofiletismo de Soleomorpha ("Soleidae"). PhD. Thesis, Museu de Zoologia da Universidade de São Paulo, São Paulo.

Ramos RTC (2003a) Family Achiridae. In: Reis RE, Kullander SO and Ferraris Jr CJ (eds) Checklist of the freshwater fishes of South and Central America. Edipucrs, Porto Alegre, pp 606-669.

Ramos RTC (2003b) Systematic review of *Apionichthys* (Pleuronectiformes, Achiridae), winth description of four new species. Ichthyol Explor Freshwaters 14:97-126.

Reeder TW (1995) Phylogenetic relationships among phrynosomatid lizards as inferred from mitochondrial ribosomal DNA sequences: Substitutional bias and information content of transitions relative to transversions. Mol Phylogenet Evol 4:203-222.

Ronquist F and Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Sakamoto K (1984) Interrelationships of the family Pleuronectidae (Pisces, Pleuronectiformes). Mem Fac Fish Hokkaido Univ 31:95-215.

Shimabukuro-Dias CK, Oliveira C, Reis RE and Foresti F (2004) Molecular phylogeny of the armored catfish family Callichthyidae (Ostariophysi, Siluriformes). Mol Phylogenet Evol 32:152-163.

Swofford DL (2002) PAUP* - Phylogenetic analysis using parsimony (*and other methods), v. 4b10. Sinauer, Sunderland.

Templeton AR (1983) Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the humans and apes. Evolution 37:221-244.

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

Xia X and Xie Z (2001) DAMBE: Data analysis in molecular biology and evolution. J Heredity 92:371-373.

Associate Editor: Maria Iracilda Sampaio