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Phylogenetic Relationships of Pleuronectiformes Based on Molecular Evidence

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Phylogenetic analyses based on nucleotide sequence data of the 12S and 16S mitochondrial ribosomal genes were performed for representatives of the Pleuronectiformes (flatfishes). Two parsimony analyses with percomorphs and basal acanthopterygians designated as outgroups were conducted; equal weighting of all nucleotides and loop regions analyzed for transversions only. A bayesian analysis was performed under the likelihood model TrN+I+Γ. A monophyletic Pleuronectiformes was resolved in all analyses. Results are consistent with the current higher level classification, recognizing the suborders Psettodoidei and Pleuronectoidei. Psettodoidei consists of a single family, Psettodidae, that is sister to all other flatfishes. Within the Pleuronectoidei three clades are identified. Bothids plus paralichthyids (in part) form a clade in all analyses. A solelike clade containing citharids, cynoglossids, samarids, soleids, *Trinectes* (Achiridae), and *Poecilopsetta* (Poecilsopsettidae), is identified in the parsimony analyses. However, a solelike clade was not recovered in the bayesian analysis. A flounderlike clade is identified containing Pleuronectidae and Paralichthyidae (in part) in all analyses. The position of *Scophthalmus* (Scophthalmidae) differed among all analyses. The results further support the conclusion that eye position is not a useful source of information for the classification of flatfishes. This study provides a new framework for future studies.

PLEURONECTIFORMES (flatfishes) are remarkable because of their dramatic metamorphosis from bilaterally symmetrical larvae to laterally compressed adults with both eyes on one side of the head. Monophyly of the Pleuronectiformes is supported by three morphological synapomorphies: (1) migration of one eye during ontogeny; (2) anterior position of the dorsal fin origin; and (3) presence of a *recessus orbitalis*, an organ that allows the eyes to protrude above the surface of the body (Chapleau, 1993). The order is comprised of two suborders, suborder Psettodoidei containing a single family, Psettodidae, with one genus and two extant species, and suborder Pleuronectoidei containing the remaining 13 families, 122 genera, and approximately 570 extant species (Chapleau, 1993; Nelson, 1994). The flatfishes are currently included within the Percomorpha (sensu Johnson and Patterson, 1993). However, relationships within the Percomorpha are largely unresolved, and the sister group to the Pleuronectiformes is unknown.

There have been many hypothesized phylogenies, and classifications of the Pleuronectiformes (Berendzen, 1998). Chapleau (1993) conducted the first comprehensive cladistic analysis of pleuronectiform relationships based on morphological characters (Fig. 1B). He questioned monophyly of a number of families and subfamilies and proposed the following classification: (1) inclusion of all families except Psettodidae in the Pleuronectoidei; (2) elevation of subfamilies Pleuronectinae, Samarinae, Rhombosoleinae, and Poecilopsettinae (Pleuronectidae) to familial level (Chapleau and Keast, 1988); and (3) elevation of Achirinae and Soleinae (Soleidae) to familial level (Chapleau and Keast, 1988). Chapleau (1993) concluded that classifying flatfishes as right- and left-eyed founders and soles is ''simplistic and phylogenetically misleading'' (p.535) and stressed a need for further studies.

Cooper and Chapleau (1998a) revised the dataset of Chapleau (1993) and included Paralichthodidae, a monotypic family and phylogenetically problematic taxon. Their results resolved Chapleau's (1993) large polytomy (Fig. 1B), but they noted the branch uniting *Citharus* was not well supported (Fig. 1C). In a separate study, Hoshino and Amaoka (1998) concluded that *Tephrinectus sinensis*, a monotypic genus, was sister to Chapleau's (1993) clade containing Rhombosoleidae, Poecilopsettidae, Samaridae, Achiridae, Soleidae, and Cynoglossidae.

The use of additional datasets, primarily DNA data, is necessary to help elucidate unresolved relationships. Herein, we examine phylogenetic relationships of flatfishes using mitochondrial rRNA sequences. The objective of this study was to use the 12S and 16S mitochondrial ribosomal DNA to elucidate relationships within Pleuronectiformes. In addition, the utility of these

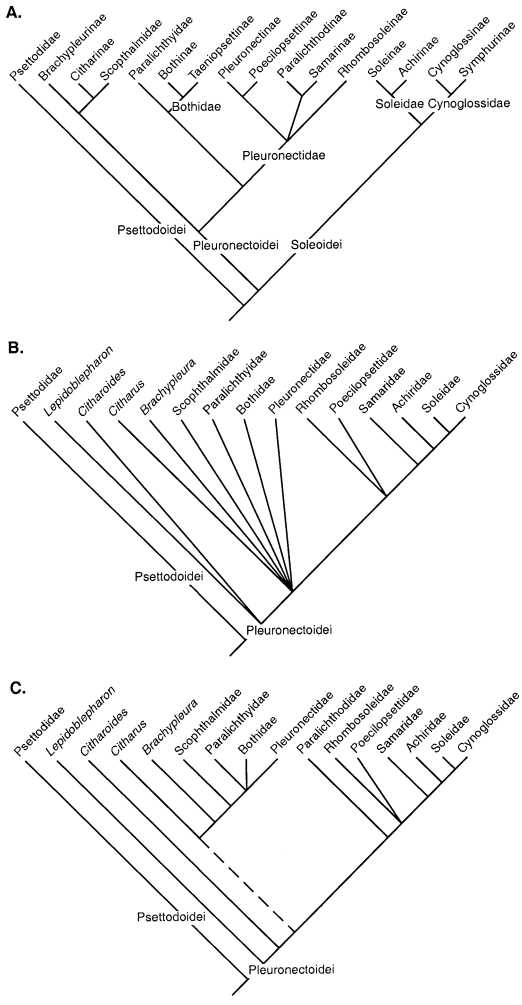

Fig. 1. (A) The Regan-Norman model of pleuronectiform relationships by Hensley and Ahlstrom (1984). (B) Hypothesis of relationships among Pleuronectiformes proposed by Chapleau (1993). (C) Hypothesis of relationships among Pleuronectiformes by Cooper and Chapleau (1998a).

data for resolving relationships among subgroups of the Pleuronectiformes was explored.

Materials and Methods

Material examined.—Tissue samples were obtained from specimens representing four bothid, two citharid, two cynoglossid, nine paralichthyid, 15 pleuronectid, two samarid, and four soleid genera, and a single genus for Achiridae, Poecilopsettidae, Psettodidae, and Scophthalmidae. *Perca* (Percidae), *Pterois* (Scorpaenidae), *Melichthys* (Balistidae), *Scopeloberyx* (Melamphaidae), *Beryx* (Berycidae), and *Zeus* (Zeidae) were used as outgroups. See Appendix 1.

DNA protocols.—DNA was extracted using QIAamp™ tissue extraction kits (Qiagen). A 600 bp fragment of the 16S ribosomal mtDNA gene was amplified using primers 16Sa-L and 16Sb-H and an 830 bp fragment of the 12S ribosomal mtDNA gene was amplified using primers Phe2-L and 12Sb-H (Table 1). PCR reactions were performed on a Perkin-Elmer 480 thermal cycler in a total volume of 100 μl containing 1.0 μg of DNA, 2 μM of each primer, 10X Taq salts (Perkin-Elmer Cetus Corp.), 10 mM of each dNTP, and 0.5 μl of Amplitaq™ DNA polymerase (Perkin-Elmer Cetus Corporation) using the following thermal profile: denaturing at 94 C for 30 sec, annealing at 55 C for 30 sec, and extension at 70 C for 2 min and 30 sec for 35 cycles. Amplified products were gel purified using a 0.8% NuSieveGTG® (FMC) low melt agarose electrophoresed for one hour at 80–90 V. Target bands were excised from the gel and the products were recovered using QIAquick™ gel extraction kits (Qiagen).

PCR products were sequenced using manual and automated methods. Manual sequencing followed Hillis et al. (1996) with primers 16Sb-H, 16S 3173, and 16S 2939 (Table 1) using Promega fmol® DNA cycle sequencing kits and γ^{32}P dATP end-labeled primers. For automated sequencing, PCR products were reamplified using the following profile: denaturing at 96 C for 30 sec, annealing at 50 C for 15 sec, and extension at 60 C for 4 min for 26 cycles and sequenced with the remaining primers (Table 1) on an ABI 310 automated sequencer using ABI Prism™ dye terminator cycle sequencing kits. Light and heavy strands for most of both fragments were sequenced. All sequences were deposited in GenBank (Appendix 1).

DNA data and alignment protocols.—The accuracy of base determination was checked by comparison of light and heavy strands. Sequences were spliced together to arrive at a consensus sequence (light strand) for each taxon using the computer program Sequence Navigator 1.01 (Applied Biosystems). Initial alignment of sequences for all taxa was made using the CLUSTAL pairwise alignment algorithm with the default settings in Sequence Navigator 1.01 (Applied Biosystems). Secondary structure was identified using published secondary structure models of the 16S and 12S genes for *Cyprinus carpio* (Van de Peer et al., 1994; de Rijk et al., 1994). Loop and stem regions of the secondary structure were identified as separate categories of data for analysis. The stem regions were checked for base pair complementarity and the alignments in the loop regions were manually

TABLE 1. SEQUENCING (S) AND AMPLIFICATION (A) PRIMERS USED IN THIS STUDY.

Name	Sequence	Strand	Use
	Mitochondrial 12S Gene		
Phe2-L[a]	5′ AAAGCATAACACTGAAGATGTTAAGATG 3′	light	A, S
12Sb-H[b]	5′ AGGAGGGTGACGGGCGGTGTGT 3′	heavy	A, S
12Sd-L[c]	5′ GGGTTGGTAAATCTCGTGC 3′	light	S
12Sd-R	5′ GCTGGCACGAGTTTTACCGGCC 3′	heavy	S
12Sa-H	5′ GTATCTAATCCCAGTTTG 3′	heavy	S
12Sa-L[b]	5′ AAACTGGGATTAGATACCCCACTA 3′	light	S
	Mitochondrial 16S Gene		
16Sa-L[d]	5′ CGCCTGTTTACCAAAAACATCGCCT 3′	light	A, S
16Sb-H[d]	5′ CCGGTCTGAACTCAGATCACGT 3′	heavy	A, S
16S 3173	5′ CGCTGTTATCCCTAGAGTAA 3′	heavy	S
16S 2939	5′ GCTTCATAGGTCTTCTCGTC 3′	heavy	S

[a] *Oncorhynchus mykiss* position 946–965.
[b] Modified from Kocher et al. (1989).
[c] Modified 503 primer of John Patton, Washington University. *Oncorhynchus mykiss* position 1216–1233.
[d] See Palumbi (1996).

adjusted (Kjer et al., 1994). Bulges, bases within stem regions not involved in base pair complementarity, were included with the stems and not as a separate category for analysis. The number of variable and parsimony-informative sites, and frequencies of transitions and transversions were calculated for the 16S and 12S aligned sequences using PAUP* 4.0b4a (D. L. Swofford, Sinauer Associates, Sunderland, MA, 1999). Uncorrected (p) distance coefficients were calculated for all pairwise comparisons among taxa for both the 16S and 12S fragments using PAUP* 4.0b4a (D. L. Swofford, Sinauer Associates, Sunderland, MA, 1999). Site saturation in loop and stem regions was assessed by plotting the uncorrected (p) distances against the total number of transitions and transversions for each pairwise comparison.

Phylogenetic analyses.—Parsimony analyses were performed using the heuristic search option, 1000 random addition sequence replicates, and tree-bisection-reconnection algorithm in PAUP* 4.0b4a (D. L. Swofford, Sinauer Associates, Sunderland, MA, 1999). *Perca*, *Pterois*, *Scopeloberyx*, *Beryx*, *Melichthys*, and *Zeus* were designated as outgroups. Approximately 290 nucleotides from the 3′ end of the 12S fragment were missing for all outgroup taxa. One hundred and twenty-one nucleotides from loop regions of the 16S fragment and 121 nucleotides from loop regions of the 12S fragment were removed from all analyses because homology of these characters could not be determined. Two parsimony analyses were performed, the first with all nucleotides weighted equally and the second with loop regions analyzed for transversions only. This weighting was done to account for evidence of saturation of transitions in the more variable loop regions. Parsimony trees were evaluated using summary values reported by PAUP* (e.g., tree length, ensemble consistency index). Support for internodes was evaluated by calculating decay indices (Bremer, 1988, 1994) using TreeRot (Sorensen, 1996). Bootstrap values (Felsenstein, 1985) were calculated using a heuristic search and 1000 bootstrap pseudoreplications.

Modeltest (Posada and Crandall, 1998) was used to determine the parameters for a hierarchy of likelihood models. The model TrN+I+Γ provided the best explanation of the data as determined by the likelihood ratio test (Posada and Crandall, 2001). A maximum likelihood analysis was not performed because of computational constraints caused by the size and complexity of the dataset. A bayesian analysis was performed to estimate the phylogeny under the likelihood model TrN+I+Γ determined by Modeltest. Analysis of the dataset was carried out using Mr. Bayes 2.01 (Huelsenbeck and Ronquist, in press). Markov chains Monte Carlo were run for 2,000,000 generations, and trees were sampled every 100 generations. Branch lengths of sampled trees were saved. The burnin was estimated at 20,000 generations where the Markov chain Monte Carlo reached stationarity leaving 19,800 trees for analysis. The posterior probabilities of trees and tree parameters were estimated from this distribution.

RESULTS

DNA sequence data were obtained for 482 aligned bases of the 16S mtRNA gene and 726

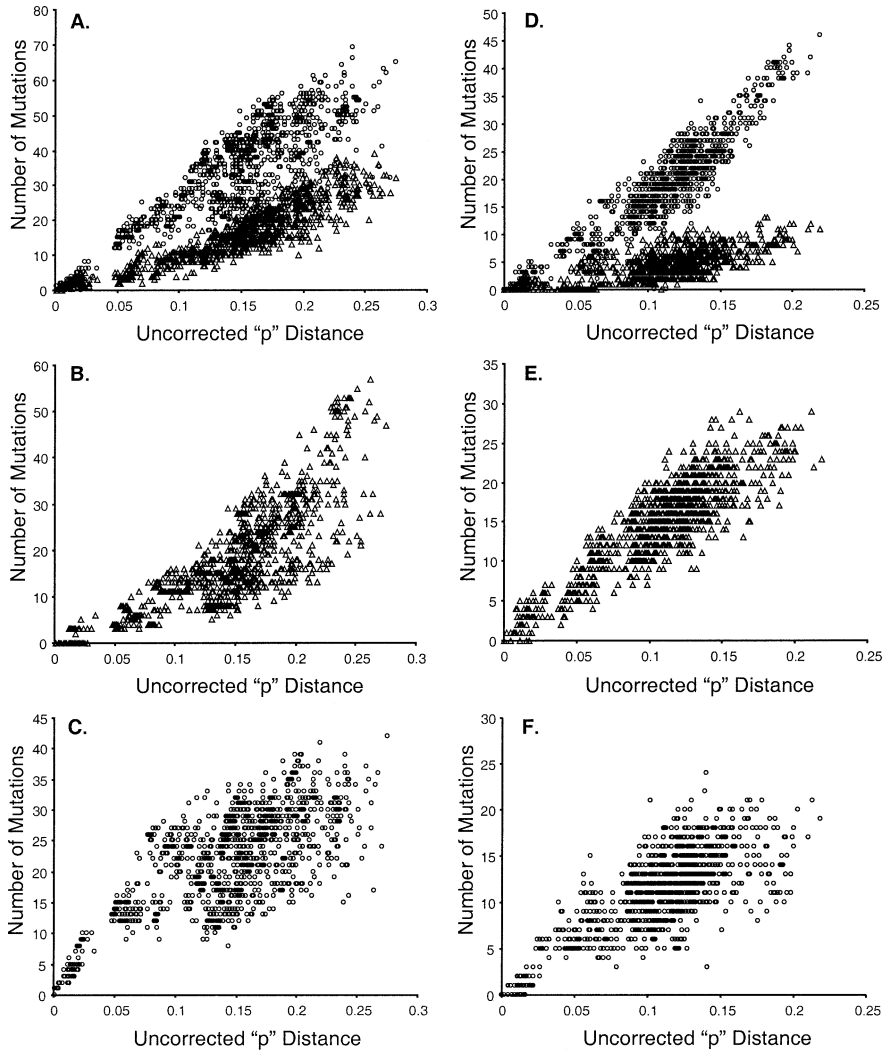

Fig. 2. Scatter-plots of the number of mutations versus uncorrected (p) distance coefficients in pairwise comparisons. (A) Transversions and transitions in 12S stem regions. (B) Transversions in 12S loop regions. (C) Transitions in 12S loop regions. (D) Transversions and transitions in 16S stem regions. (E) Transversions in 16S loop regions. (F) Transitions in 16S loop regions. (o) Transitions. (△) Transversions.

aligned bases of the 12S mtRNA gene for 50 taxa. The aligned dataset is available from the senior author. Stem regions of the 16S fragment contained 286 nucleotides of which 142 were variable and 95 were parsimony informative in both the weighted and unweighted analyses. Loop regions contained 196 nucleotides of which 114 were variable; 88 were parsimony informative in the unweighted analysis and 84 in the weighted analysis. Stem regions of the 12S fragment contained 411 nucleotides of which 233 were variable and 182 were parsimony informative in both the weighted and unweighted analyses. Loop regions contained 315 nucleotides of which 219 were variable; 154 were parsimony informative in the unweighted analysis and 139 in the weighted analysis.

Scatter-plots of uncorrected (p) distance coefficients versus number of mutations are presented in Figure 2. A linear relationship between the number of mutations and genetic distance in both the 16S and 12S fragments suggests that there was no site saturation for transitions or transversions in the stem regions (Fig. 2A, D). Similarly there was no evidence of site saturation of transversions in loop regions (Fig. 2B, E). A plateau for the plot of loop transitions versus genetic distance in both the 16S and 12S fragments suggests that loop transitions are saturated and thus inappropriate for phy-

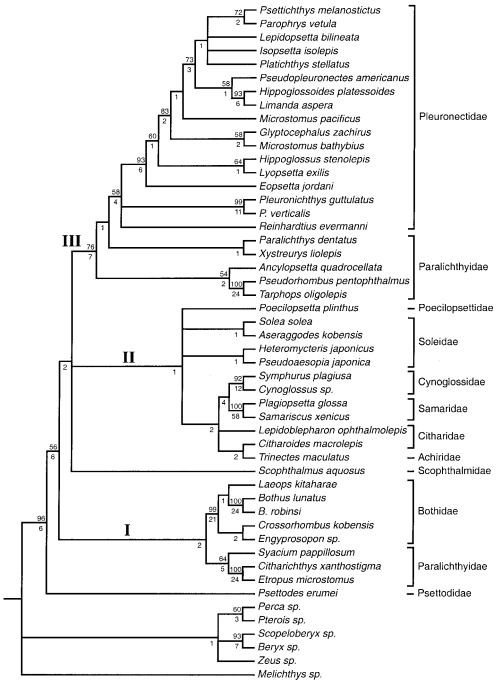

Fig. 3. The strict consensus tree resulting from 32 equally most-parsimonious trees with all nucleotides weighted equally. Numbers above nodes indicate bootstrap values (1000 pseudoreplicates) and numbers below nodes indicate Bremer decay indices. TL = 3391, CI = 0.3151, HI = 0.6849, and RI = 0.4494.

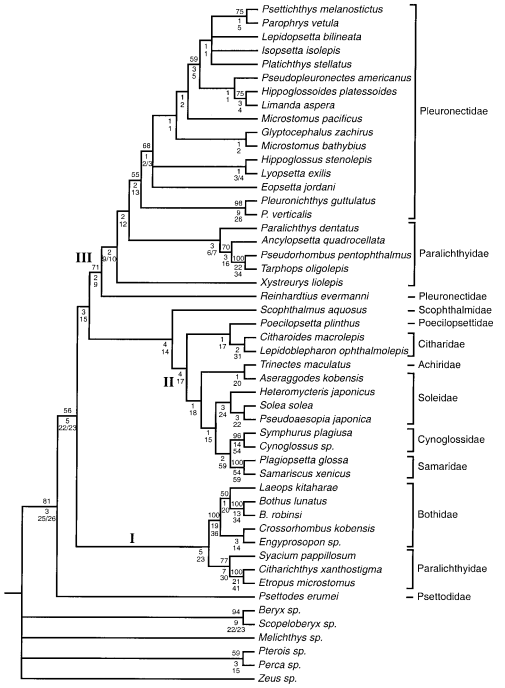

Fig. 4. The strict consensus tree resulting from eight equally most-parsimonious trees with loop regions analyzed for transitions only. Numbers above nodes indicate bootstrap values (1000 pseudoreplicates), numbers below nodes indicate Bremer decay indices, and bottom numbers indicate branch lengths. TL = 2560, CI = 0.2960, HI = 0.7040, and RI = 0.4667.

logenetic analysis (Fig. 2C, F). Each gene region was analyzed for saturation as a whole, no division of stems and loops, and no evidence of site saturation of transitions was detected. Thus, analysis of a whole gene for saturation can obscure its presence in regions that are less constrained and evolve rapidly.

Unweighted parsimony analyses.—Parsimony analysis of the unweighted nucleotide data resulted in 32 equally most-parsimonious trees (TL = 3391, CI excluding uninformative characters = 0.3151, RI = 0.4494). The strict consensus of these trees is presented in Figure 3. The Pleuronectiformes were monophyletic with a bootstrap value (BV) of 96 and a Bremer decay index (BDI) of 6. *Psettodes* were sister to all other flatfishes. Remaining taxa were divided into three parts to facilitate discussion. Part I contains Bothidae and Paralichthyidae (in part) and was monophyletic in all analyses. Part II contains *Trinectes*, Citharidae, Cynoglossidae, *Poecilopsetta*, Samaridae, and Soleidae and was monophyletic in some analyses. Part III contains Pleuronectidae and remaining Paralichthyidae and was monophyletic in all analyses. *Scophthalmus* formed a tricotomy with parts II and III.

Weighted parsimony analyses.—Parsimony analysis of the nucleotide data excluding loop transitions resulted in eight equally most-parsimonious trees (TL = 2560, CI excluding uninformative characters = 0.2960, RI = 0.4667). The strict consensus of these trees is presented in Figure 4. Deep nodes were stable among all equally most-parsimonious trees. Differences were found within the relationships of pleuronectid genera *Isopsetta*, *Lepidopsetta*, and *Platichthys*. Branch lengths (BL) are provided for the weighted analysis as an additional estimator of nodal support. The Pleuronectiformes were monophyletic (BV = 81, BDI = 3, BL = 25/26). Parts I, II, and III represent the same taxa as in Figure 3. The relationships at the basal portion of the tree (*Psettodes* and part I) were the same as the unweighted analysis (Fig. 3) with similar bootstrap values and Bremer decay indices. *Scophthalmus* was resolved as sister to part II. Within part III, the representative pleu-

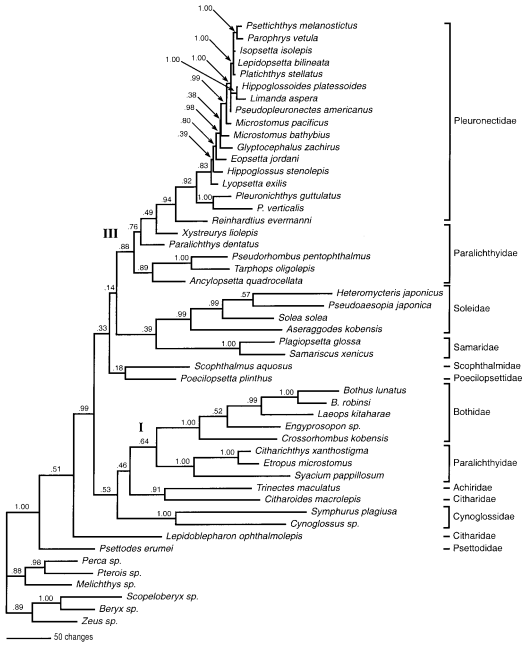

Fig. 5. Fifty percent majority rule tree produced by a bayesian analysis under TrN+I+Γ. Posterior probabilities from 19,800 trees are indicated on the branches.

ronectid taxa did not form a monophyletic group.

Bayesian analyses.—Results from Modeltest (Posada and Crandall, 1998) indicated the model TrN+I+Γ provided the best fit to the data. The bayesian analysis produced 20,000 trees of which 200 were discarded as burnin. This left 19,800 trees to estimate topology, branch lengths, and tree parameters. The percentage of times a node occurs within these 19,800 trees was interpreted as the posterior probability of the node. Branch lengths of the sampled trees were calculated and used to construct the tree shown in Figure 5. Estimated model parameters were -ln l = −14438.67 ± 85.37, A = 0.300696 ± 0.000104, C = 0.261217 ± 0.000104, G = 0.202361 ± 0.000091, T = 0.235726 ± 0.000068, α = 0.331627 ± 0.000212. The Pleuronectiformes were monophyletic (posterior probability = 1.00). *Psettodes* was sister to all other flatfishes. Part I contains Bothidae and Paralichthyidae (in part) and part III contains Pleuronectidae and the remaining Paralichthyidae. The members of part II identified in the parsimony analyses did not form a monophyletic group in the bayesian analysis. Many of the relationships among these taxa differed. *Scophthalmus* was resolved as sister to *Poecilopsetta*.

Discussion

Although flatfishes have long been regarded as a natural group (Regan, 1910; Norman, 1934; Hubbs, 1945), only recently were supporting synapomorphies explicitly defined (Chapleau, 1993). The results of molecular analyses presented herein strongly support monophyly of the Pleuronectiformes. Our analyses contradict the traditional view of relationships among flatfishes as outlined in the Regan-Norman model of Hensley and Ahlstrom (1984; Fig. 1A) and are consistent with the higher level classification of Chapleau (1993) and Cooper and Chapleau (1998a; Fig. 1B–C). Our results support the suborders Psettodoidei and Pleuronectoidei, although the phylogenetic relationships of the families within the Pleuronectoidei differ.

Psettodidae have been considered the most primitive group of the pleuronectiforms (Norman, 1934; Hensley and Ahlstrom, 1984; Chapleau, 1993). The results of all molecular analyses strongly support *Psettodes* (Psettodidae, Psettodoidei) as sister to all other flatfishes (Pleuronectoidei).

Within the Pleuronectoidei, we identified part I (Figs. 3–5), a clade containing members of the sinistral family Bothidae plus members of the paralichthyid *Cyclopsetta* group (Hensley and Ahlstrom, 1984); part II (Figs. 3–4), a clade containing citharids, cynoglossids, samarids, soleids, *Trinectes* (Achiridae), and *Poecilopsetta* (Poecilopsettidae); and part III (Figs. 3–5) a clade containing Pleuronectidae (sensu Chapleau and Keast, 1988) and remaining members of the Paralichthyidae.

Part I.—In all analyses the monophyly of part I was recovered. However, the position of this group in each analysis differed; all analyses contradict Chapleau (1993) and Cooper and Chapleau (1998a). Although monophyly of bothid taxa was supported in all analyses (Figs. 3–5), low taxon sampling did not allow us to comment on the interrelationship of the group. The Paralichthyidae, a generalized group of left-eyed flounders, have been recognized as polyphyletic (Hensley and Ahlstrom, 1984; Chapleau, 1993), but alternate hypotheses of relationships of all included taxa have not been proposed. Hensley and Ahlstrom (1984) identified a monophyletic *Cyclopsetta* group containing the paralichthyid genera *Citharichthys*, *Cyclopsetta*, *Etropus*, and *Syacium* and suggested this group was closely related to the bothids. Chapleau (1993) also recognized the monophyly of the *Cyclopsetta* group. Although this study lacked a representative of the genus *Cyclopsetta*, the results were consistent

with Hensley and Ahlstrom (1984) and Chapleau (1993) supporting a monophyletic group containing *Citharichthys*, *Etropus*, and *Syacium* that was more closely related to bothids than remaining paralichthyids (part I, Figs. 3–5).

Scophthalmidae.—The position of *Scophthalmus*, Scophthalmidae, differed among all our analyses (Figs. 3–5). Chapleau (1993) was also unable to determine the position of Scophthalmidae (Fig. 1B); however, Cooper and Chapleau (1998a; Fig. 1C) resolved it as sister to a polytomy containing Bothidae, Paralichthyidae, and Pleuronectidae. The lack of resolution of *Scophthalmus* does not add further understanding of the phylogenetic position of this group.

Part II.—In both parsimony analyses, the monophyly of part II, containing the solelike flatfishes; citharids, cynoglossids, samarids, soleids, *Trinectes*, and *Poecilopsetta*, was recovered (Figs. 3–4). However, most relationships within part II in the parsimony analyses were not strongly supported. This was reflected in the results of the bayesian analysis (Fig. 5), which did not resolve a monophyletic part II.

Chapleau and Keast (1988) concluded that the Pleuronectidae (sensu Norman, 1934) were not monophyletic and elevated the subfamilies. Later, Chapleau (1993) hypothesized that Samaridae and Poecilopsettidae, former pleuronectid subfamilies, were more closely related to the solelike flatfishes than pleuronectids. The results of this study were consistent with this hypothesis, but differed in the relationships among families. Both parsimony analyses placed *Poecilopsetta* (Poecilopsettidae) within part II, although the relationships differed. The bayesian analysis resolved *Poecilopsetta* as sister to *Scophthalmus* and more closely related to part I than the solelike flatfishes.

All analyses strongly supported monophyly of the samarid taxa and monophyly of the cynoglossid taxa. Both parsimony analyses (Figs. 3–4) resolved the samarids as sister to the cynoglossids, although this relationship was not well supported. In contrast, the bayesian analysis resolved samarids as sister to the soles. A close relationship of the samarids to the soles is consistent with Chapleau (1993) and Cooper and Chapleau (1998a) (Fig. 1B–C). However, in the bayesian analysis the cynoglossids were found to be sister to *Trinectes* + *Citharoides* + part I. This relationship is contrary to any previous hypothesis. None of the molecular analyses were consistent with Chapleau (1993) and Chapleau and Keast (1988) who recognized soleids as the sister to Cynoglossidae and samarids as sister to Achiridae plus Soleidae and Cynoglossidae.

Soleidae are considered a monophyletic group (Chapleau and Keast, 1988). Four of 20 genera were included in this study, but were not monophyletic in the parsimony analyses (Figs. 3–4). However, the bayesian analysis supported monophyly of the included soleid taxa (Fig. 5). There was no strong evidence to question the monophyly of Soleidae.

A single representative of the Achiridae, *Trinectes maculatus*, was included in this analysis. In both parsimony analyses *Trinectes* was included in part II. In the unweighted parsimony and bayesian analyses *Trinectes* was resolved as sister to the citharid genus *Citharoides*. In the bayesian analysis *Trinectes* + *Citharoides* was sister to part I. This relationship is contrary to any previous hypotheses. The weighted parsimony analysis is more consistent with previous hypotheses (Fig. 1A–C), which recognized a close relationship between Achiridae and Soleidae.

Citharidae is the only flatfish family to contain both right-eyed (*Brachypleura* and *Lepidoblepharon*) and left-eyed (*Citharoides* and *Citharus*) genera. Chapleau (1993) concluded the group was polyphyletic and the genera *Lepidoblepharon* and *Citharoides* were basal pleuronectoids (Fig. 1B). Our analyses included both the right-eyed genus *Lepidoblepharon* and the left-eyed genus *Citharoides*. The unweighted parsimony and bayesian analyses (Figs. 3, 5) did not resolve a monophyletic Citharidae. In both analyses, *Citharoides* was sister to *Trinectes*; however, the position of this clade differed. Interestingly, in the bayesian analysis *Lepidoblepharon* was sister to the remaining Pleuronectoidei. This basal position is consistent with Chapleau (1993) and Cooper and Chapleau (1998a). In the weighted parsimony analysis the citharid taxa formed a monophyletic group. However, the position of the citharid taxa contradicts Chapleau (1993) and Cooper and Chapleau (1998a) and suggests that they are more closely related to solelike flatfishes than bothids or pleuronectids. We were unable to draw any definitive conclusions on the position of *Citharoides* and *Lepidoblepharon* within the Pleuronectoidei.

Part III.—In all analyses, monophyly of part III, representing a generalized flounderlike group containing the Pleuronectidae (sensu Chapleau and Keast, 1988) and remaining members of the Paralichthyidae, was recovered (Figs. 3–5). In the unweighted parsimony and bayesian analyses, a monophyletic Pleuronectidae was recovered but was not recovered in the weighted parsimony analysis.

In all analyses, the paralichthyid genera *Ancylopsetta*, *Paralichthys*, *Pseudorhombus*, *Tarphops*, and *Xystreurys* were included in part III. However, these genera did not form a monophyletic paralichthyid group. *Pseudorhombus* and *Tarphops*, members of the *Pseudorhombus* group (sensu Amaoka, 1969), formed a strongly supported group followed by *Ancylopsetta* in all analyses. *Paralichthys* and *Xystreurys* were then sister to this group; however, the relationships differed among analyses. Paralichthyidae has been considered polyphyletic (Hensley and Ahlstrom, 1984; Chapleau, 1993), and based on our results it is clear that the paralichthyid genera have a close association with the pleuronectids.

Cooper and Chapleau (1998b) established a new classification within Pleuronectidae (sensu Chapleau and Keast, 1988). Our study included 13 of 21 genera recognized by Cooper and Chapleau (1998b). However, low taxon sampling did not allow us to comment extensively on the interrelationships of the family. The basal position of *Reinhardtius*, sister to remaining pleuronectids, in the unweighted parsimony and bayesian analyses was consistent with their hypothesis. Within the Pleuronectidae, monophyly of the clade containing *Pleuronichthys guttulatus* and *Pleuronichthys verticalis* was strongly supported in all analyses, which is consistent with Cooper and Chapleau (1998b). However, the position of this group within the Pleuronectidae differed in our analyses. Cooper and Chapleau (1998b) also hypothesized a close relationship between *Microstomus* and *Glyptocephalus* (sensu Cooper and Chapleau, 1998b), which was observed in our analyses. However, *Microstomus* (sensu Cooper and Chapleau, 1998b) was never found to be monophyletic. Expanded studies including paralichthyids and pleuronectids are necessary to determine relationships within the Pleuronectidae with respect to the work of Cooper and Chapleau (1998b).

Conclusion.—Traditionally flatfishes have been classified as right- and left-eyed flounders, and right- and left-eyed soles. Flounders and soles were hypothesized to have arisen from a *Psettodes*-like ancestor and within each group a left- and right-eyed form was proposed to have arisen from a common ancestor. As more knowledge was gained, the understanding of the relationships among flatfishes became more complex. However, sidedness remained an important characteristic for classification. Chapleau (1993) was the first to offer an alternate hypothesis. He concluded that right and left sidedness was derived multiple times within flatfishes and eye position is not a clear indicator of relationships within the group. The results of this study were congruent with Chapleau's conclusion that sidedness is not phylogenetically informative. Our results provide new directions for future research on the relationships within flatfishes.

The Pleuronectiformes offer a unique set of challenges and problems in trying to resolve their relationships and generate a classification that reflects their relationships. Flatfishes have a reduced morphology that makes them difficult to study morphologically. Unfortunately, the use of exemplar taxa is unavoidable in phylogenetic analyses of large clades. This is especially true for the Pleuronectiformes which are a large, complex group with a worldwide marine distribution. Representative taxa of all groups are difficult to obtain. Incomplete taxon sampling can be a problem with phylogeny estimation (Swofford et al., 2001), which may be reflected in the inconsistencies among our analyses. However, our study offers the first comprehensive molecular analysis of relationships among flatfishes and provides new insight into relationships among flatfishes. Additional taxa and data will be necessary to provide a robust phylogenetic hypothesis of pleuronectiform relationships.

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APPENDIX 1 SPECIMENS EXAMINED Species used in this study are listed with higher level classification of the Pleuronectiformes (following Chapleau, 1993 and Cooper and Chapleu, 1998b). Institutional abbreviations are as follows: AMS = Australian Museum, KU = University of Kansas; USNM = Smithsonian Institution, United States National Museum; UW = University of Washington. The catalog number and tissue number are given after each species with GenBank accession numbers for 12S (first) and 16S (second) in parentheses.

Pleuronectiformes		
Psettodoidei		
Psettodidae		
Psettodes erumei	USNM 345211, USNM T4736	(AF488518, AF488468)
Pleuronectoidei		
Pleuronectidae		
Hippoglossinae		
Reinhardtius evermanni	KU uncat, KU T379	(AF488475, AF488425)
Hippoglossus stenolepis	UW 041682, KU T3103	(AF488483, AF488433)
Eopsettinae		
Eopsetta jordani	KU 23700, KU T596-1	(AF488476, AF488426)
Hippoglossoidinae		
Hippoglossoides platessoides	KU uncat, KU T368	(AF488477, AF488427)
Lyopsettinae		
Lyopsetta exilis	KU 25749, KU T422	(AF488484, AF488434)
Pleuronectinae		
Psettichthyini		
Psettichthys melanostictus	KU 23697, KU T583	(AF488485, AF488435)
Isopsettini		
Isopsetta isolepis	KU 25758, KU T431	(AF488481, AF488431)
Microstomini		
Glyptocephalus zachirus	KU 23740, KU T588	(AF488486, AF488436)
Lepidopsetta bilineata	KU uncat, KU T378	(AF488479, AF488429)
Microstomus bathybius	KU uncat, KU T2268	(AF488490, AF488440)
Microstomus pacificus	KU 23703, KU T592	(AF488480, AF488430)
Pleuronichthys guttulatus	KU uncat, KU T484	(AF488487, AF488437)
Pleuronichthys verticalis	KU uncat, KU T445	(AF488489, AF488439)
Pleuronectini		
Limanda aspera	KU uncat, KU T385	(AF488491, AF488441)
Parophrys vetula	KU 23756 KU T587	(AF488488, AF488438)
Platichthys stellatus	KU 25755, KU T428	(AF488482, AF488432)
Pseudopleuronectes americanus	KU uncat, KU T365	(AF488478, AF488428)
Poecilopsettidae		
Poecilopsetta plinthus	KU 27256, KU T2472	(AF488515, AF488465)
Samaridae		
Plagiopsetta glossa	KU 27257, KU T2470	(AF488516, AF488466)
Samariscus xenicus	KU 27266, KU T2482	(AF488517, AF488467)
Citharidae		
Brachypleuronae		
Lepidoblepharon ophthalmolepis	KU 27263, KU T2495	(AF488514, AF488464)
Citharinae		
Citharoides macrolepis	KU 27264, KU T2466	(AF488513, AF488463)
Bothidae		
Bothus lunatus	KU uncat, KU T154	(AF488508, AF488458)
Bothus robinsi	KU 27035, KU T1168	(AF488509, AF488459)
Crossorhombus kobensis	KU 27267, KU T2485	(AF488506, AF488456)
Engyprosopon sp.	KU 27271, KU T2499	(AF488510, AF488460)
Laeops kitaharae	KU 27272, KU T2505	(AF488511, AF488461)

Appendix 1 Continued.

Paralichthyidae		
Ancylopsetta quadrocellata	KU 22951, KU T10	(AF488500, AF488450)
Citharichthys xanthostigma	KU uncat, KU T450-1	(AF488499, AF488449)
Etropus microstomus	KU 27165, KU T1505	(AF488502, AF488452)
Paralichthys dentatus	KU 27078, KU T1239	(AF488501, AF488451)
Pseudorhombus pentophthalmus	KU 27258, KU T2479	(AF488505, AF488455)
Syacium pappillosum	KU 27108, KU T1533	(AF488503, AF488453)
Tarphops oligolepis	KU 27269, KU T2496	(AF488507, AF488457)
Xystreurys liolepis	KU uncat, KU T465	(AF488504, AF488454)
Scophthalmidae		
Scophthalmus aquosus	KU 27107, KU T1252	(AF488512, AF488462)
Achiridae		
Trinectes maculatus	KU 22952, KU T11	(AF488496, AF488446)
Soleidae		
Aseraggodes kobensis	KU 27259, KU T2476	(AF488493, AF488443)
Heteromycteris japonicus	KU 27268, KU T2491	(AF488494, AF488444)
Pseudoaesopia japonica	KU 27273, KU T2504	(AF488495, AF488445)
Solea solea	KU 25125, KU T1846	(AF488492, AF488442)
Cynoglossidae		
Symphurinae		
Symphurus plagiusa	KU 27173, KU T1520	(AF488497, AF488447)
Cynoglossinae		
Cynoglossus sp.	KU 27260, KU T2477	(AF488498, AF488448)
Perciformes		
Percidae		
Perca sp.	KU uncat, KU T817	(AF488519, AF488469)
Scorpaeniformes		
Scorpaenidae		
Pterois sp.	USNM 334212, KU T717	(AF488520, AF488470)
Tetradontiformes		
Balistidae		
Melichthys niger	KU photo voucher, KU T105	(AF150007, AF488471)
Stephanoberyciformes		
Melamphaidae		
Scopeloberyx robustus	KU 26889, KU T276	(AF149992, AF488472)
Beryciformes		
Bericidae		
Beryx sp.	KU uncat, KU T827	(AF149995, AF488473)
Zeiformes		
Zeidae		
Zeus faber	AMS NI 1090	(AF149993, AF488474)