Molecular Phylogeny of Seven Pleuronectid Species Inferred from the Sequence of their Cytochrome Oxidase Subunit I Gene

by

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In memory of my grandfather

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Abstract

A 1213 base pair region of mitochondrial DNA corresponding to the cytochrome oxidase subunit I gene (COI) from 7 pleuronectid flatfishes (Order Pleuronectiformes) and one outgroup (*Paralichthys dentatus)* was PCR-cloned and sequenced. Pleuronectid taxa were chosen so that the main branching points in a cladogram of almost all species of Pleuronectidae (Cooper 1996) could be tested using an independent data set. Our results show that the COI sequence is highly conserved and that most of the substitutions that occur between species are synonymous changes at the third codon position. Analyses of the substitutional spectrum reveals a bias favouring the occurrence of transitions over transversions. Furthermore, transitions between C and T and transversions between A and C or T are more frequent than transitions or transversions involving G. COI sequence composition shows a bias against the occurrence of G and a preference for A at the third position of fourfold degenerate codons. Phylogenetic analyses based on all substitutions and on transversions only using parsimony and distance methods were congruent in most respects but were unable to resolve the branching order of *Pseudopleuronectes americanus* and *Limanda ferruginea* due either to a shortage of characters or homoplasy. The topology of the molecular trees provide support for the subfamily relationships in Cooper's classification (1996). However, the basal position of *Microstomus pacificus* in the molecular trees is largely incongruent with the morphology tree. This result may question the monophyletic status of the Pleuronectinae (*sensu* Cooper 1996) or may be an indication of mitochondrial introgression involving *Microstomus pacificus*.

Résumé

Une région de 1213 paires de bases de l'ADN mitochondrial correspondant à la sous-unité I de la cytochrome oxidase (COI) de sept poissons plats pleuronectidés (Ordre Pleuronectiformes) a été clonée par la méthode de la "Polymerase Chain Reaction" (PCR) et a été séquencée. Les pleuronectidés ont été choisis dans le but de vérifier, avec une source de données indépendantes, les principaux branchements d'un cladogramme des espèces de pleuronectidés obtenu à la suite d'une étude morphologique (Cooper 1996). Nos résultats montrent que la séquence de COI est hautement conservée et que la plupart des substitutions interspécifiques sont des changements synonymiques à la troisième position du codon. Les analyses du spectre de substitution ont révélé un biais en faveur des transitions relativement aux transversions. De plus, les transitions entre C et T et les transversions entre A et C ou T sont plus fréquentes que les transitions ou transversions impliquant G. La séquence COI montre un biais contre la présence de G et une préférence pour A à la troisième position des quadruples codons dégénérés. Les analyses phylogénétiques basées sur toutes les substitutions et uniquement sur les transversions en utilisant le principe de parcimonie et les méthodes de distance sont, pour l'ensemble, congruentes mais n'ont pu résoudre l'ordre des embranchements pour *Pseudopleuronectes americanus* and *Limanda ferruginae*, probablement par manque de caractères informatifs ou un suite à un haut niveau d'homoplasie. La topologie des arbres moléculaires corrobore la phylogenèse au niveau de la sous-famille obtenue par Cooper (1996). Cependant, la position basale de *Microstomus pacificus* dans les arbres moléculaires est en conflit avec sa position dans le cladogramme morphologique. Ceci peut soit remettre en question la monophilie des pleuronectinés (sensu Cooper 1996) ou soit être le résultat d'une introgression mitochondriale impliquant une espèce de *Microstomus*.

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Introduction

Over the past decade the use of DNA and amino acid sequences in the study of evolutionary relationships of organisms has become common place (Hillis and Moritz 1990; Miyamoto and Cracraft 1991). Molecular sequence data provides large numbers of precisely comparable characters for evolutionary/phylogenetic analysis (Felsenstein 1988). Although more and more phylogenetic studies use molecular sequences, the number of different genes that have been applied to phylogenetic questions is relatively small considering the possibilities (Graybeal 1994).

A large proportion of the sequences used in evolutionary studies of animals come from the mitochondrial genome. Studies of the complete mitochondrial genome from several vertebrates (Desjardins and Morais 1989; Gadaleta et al. 1989; Arnason et al. 1991; Roe et al. 1985; Tzeng et al. 1992) showed that it has a highly conserved gene content and order, is characteristically compact with very few, if any, bases separating genes and has no introns (Wolstenholme 1992).

Mitochondrial genes of vertebrates have several general characteristics which make them useful tools for phylogenetic inference: i) relative ease of amplification using universal primers and PCR techniques, ii) a higher evolutionary rate than nuclear genes and iii) a single copy number and thus freedom from complications associated with comparison of paralogous genes (Ballard and Kreitman 1995; Kumazawa and Nishida 1993; Zardoya and Meyer 1996). Vertebrate mitochondrial genomes are also characterized, for the most part, by maternal inheritance and lack of recombination (Ballard and Kreitman 1995; Kumazawa and Nishida

1993; Zardoya and Meyer 1996) features which are more useful for population level studies than for phylogenetic reconstruction. Variation between mitochondrial alleles from different populations of the same species should be small compared to variation between genes from different species or genera. For instance, cytochrome b (cytb) and mitochondrial control region sequences from rainbow fishes showed less than 1% divergence between individuals of the same species while individuals from different genera showed divergences of greater than 17% (Zhu et al. 1994). Thus, phylogenies based on mitochondrial data should not be affected by the mode of inheritance or by recombination events between mitochondrial alleles from the same species.

Several studies examining fish phylogenies across a broad range of taxonomic depth have used mitochondrial sequence data as a phylogenetic marker. Chow and Kishino (1995) used cytochrome b and ATPase sequences from eight tuna species to test the proposed interrelationships within the genus *Thunnus*. The results of this study and others using molecular data were incongruent with the morphology based phylogeny in regards to the branching order of certain species within the genus (Chow and Kishino 1995). Phylogenetic analysis of mitochondrial control region sequences from species of Pacific salmon yielded results that were largely congruent with those based on morphological characters as well as RFLP and other mitochondrial sequence data (Shedlock et al. 1992). Meyer et al. (1990) confirmed the monophyletic status of a large group of Lake Victoria cichlid species using cytochrome b and mitochondrial control region sequences. Lockhart et al. (1995) supported a revised swordtail (genus *Xiphophorus*) taxonomy based on analysis of cytochrome b and mitochondrial control region data. Zhu et al. (1994) used approximately 700 base pairs of mitochondrial sequence from 23 taxa to study the interrelationships among species rainbow fishes. Mitochondrial data have also been used to probe deeper evolutionary branches within fishes as well as the relationship between fishes and tetrapods. Normark et al. (1991) sequenced approximately 1 kilobase of mitochondrial DNA encoding portions of the COI, COII and Cytb genes from 12 species of Neopterygian fishes in order to clarify the relationship between the Teleostei, Amiidae (bow fins) and Lepisosteidae (gars). The consensus tree was largely incongruent with the morphology based phylogeny for this group. Block et al. (1993) constructed a molecular phylogeny for mackerels, billfishes and tunas in order to study the evolutionary history of endothermy in teleost fishes. Mapping of physiological strategies for endothermy onto this phylogeny suggests that endothermy has evolved at least three times within this order. Yokobori et al. (1994) examined evolutionary relationships between the lungfishes, coelacanth and tetrapods using COI amino acid sequences. All three possible relationships between these groups have been proposed by both morphology and molecular data sets. Phylogentic analysis of COI amino acid sequence supports a monophyletic lungfish coelacanth clade (Yokobori et al. 1994). However, Hedges et al. (1993) addressed the same question of lungfish, coelacanth and tetrapod relationships using approximately 3 kilobases of mitochondrial sequence including the 16s and 12s ribosomal genes. Analyses of these data support a monophyletic lungfish/tetrapod clade (Hedges et al. 1993). In this study, a 1213 base pair region of the COI gene was sequenced in order to determine the interrelationships of seven flatfish species from the family Pleuronectidae (*sensu* Chapleau and Keast 1988).

Jordan and Evermann (1898), Regan (1910), Norman (1934) and, Hubbs (1945) considered the Pleuronectidae, the right-eyed flounders, as being divided into five subfamilies: Samarinae, Rhombosoleinae, Paralichthodinae, Poecilopsettinae and, Pleuronectinae (for a more detailed description of the history of classification of Pleuronectidae see appendix I). Hensley and Ahlstrom (1984) were the first to question the monophyletic status of the Pleuronectidae based on the plesiomorphic nature of the diagnostic traits traditionally used to define the family. Furthermore, they suggested that the Pleuronectinae (*sensu* Norman 1934) was a member of a monophyletic clade (bothoid clade) that also included the Paralichthyinae, Scophthalminae, Bothinae and, *Brachylpleura*, based on a derived caudal skeleton. Chapleau and Keast (1988) and Chapleau (1993) concurred with Hensley and Ahlstrom (1984) regarding the dubious nature of the monophyletic status of the Pleuronectidae (*sensu* Norman 1934) and suggested that the four pleuronectid subfamilies be raised to the familial level (Chapleau and Keast 1988). Moreover, Chapleau (1993) obtained the bothoid clade of Hensley and Ahlstrom (1984) in only one of 18 equally parsimonious trees indicating that the definition of the bothoid clade probably needed more corroborative characters. He also found that three of the pleuronectid subfamilies (Poecilopsettinae, Samarinae and, Rhombosoleinae) formed a monophyletic group with the sole families (Achiridae, Cynoglossidae and, Soleidae). Cooper (1996) has recently demonstrated that the Pleuronectidae (*sensu* Chapleau and Keast 1988) was monophyletic based on 10 synapomorphies. In addition, he has addressed the broader and more complex issue of the interrelationships of 53 species within the family. His cladistic analysis has resulted in a new phylogenetic classification of species within the group (Table 1). This new classification is largely incongruent with the classification of Sakamoto (1984) which is based on a detailed phenetic analysis of the morphology of taxa within the

Pleuronectidae (*sensu* Norman 1934).

In this study, we have sequenced a region of the COI gene from seven pleuronectid taxa and one outgroup to verify some of the main nodes in the new classification of Cooper (1996). Taxa were chosen based on the position they occupied in the new classification (Table 1). Our molecular phylogenies are the first to be based on molecular sequence data for this group, and are compared to phylogenies based on morphology (Cooper 1996; Sakamoto 1984) for these same seven taxa (Figure 1A,B).

Table 1 Classification of the Pleuronectidae *sensu* Cooper (1996).

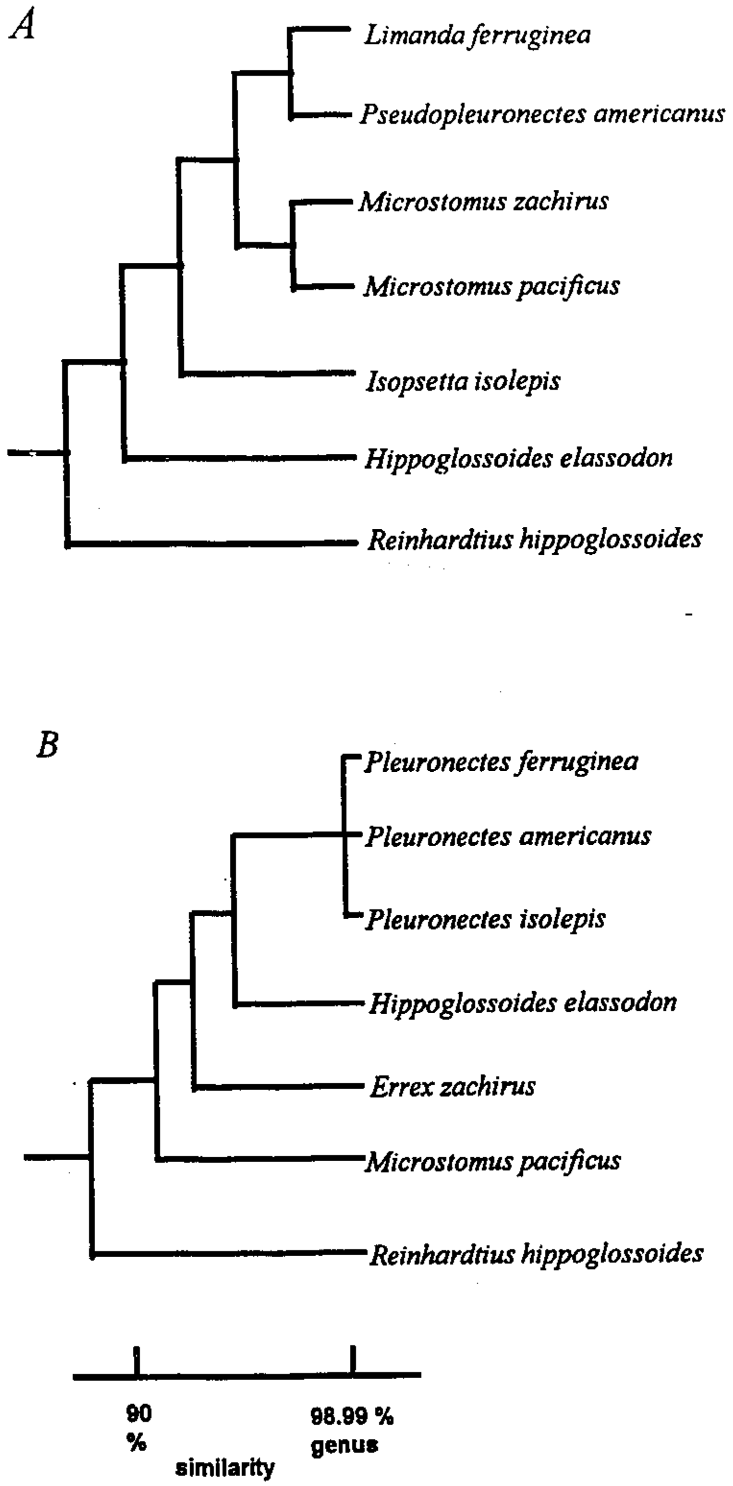
Pleuronectidae

- subfamily Hippoglossinae
  - tribe Atherestini
    - genus *Atherestes* (*A. stomias, A. evermanni*)
    - genus *Reinhardtius* (***R. hippoglossoides***)
  - tribe Hippoglossini
    - genus *Hippoglossus* (*H. hippoglossus, H. stenolepis*)
    - genus *Verasper* (*V. variegatus, V. moseri*)
    - genus *Clidoderma* (*C. asperimum*)
- subfamily Eopsettinae
  - genus *Eopsetta* (*E. grigorjewi, E. jordani*)
- subfamily Lyopsettinae
  - genus *Lyopsetta* (*L. exilis*)
- subfamily Hippoglossoidinae
  - genus *Acanthopsetta* (*A. nadeshnyi*)
  - genus *Cleisthenes* (*C. herzesteini, C. pinetorum*)
  - genus *Hippoglossoides* (***H. elassodon***, *H. platessoides, H. robustus, H. dubius*)
- subfamily Pleuronectinae
  - tribe Isopsettini
    - genus *Isopsetta* (***I. isolepis***)
  - tribe Psettichthyini
    - genus *Psettichthys* (*P. melanostictus*)
  - tribe Microstomini
    - genus *Lepidopsetta* (*L. bilineatus, L. mochigarei*)
    - genus *Dexistes* (*D. rikuzenius*)
    - genus *Pleuronichthys* (*P. coenosus, P. cornutus, P. decurrens, P. guttulata, P. ritteri, P. verticalis*)
    - genus *Microstomus* (*M. achne, M. bathybius, M. cynoglossus, M. kitaharai, M. kitt*, ***M. pacificus***, *M. stelleri*, ***M. zachirus***)
  - tribe Pleuronectini
    - genus *Parophrys* (*P. vetulus*)
    - genus *Limanda* (*L. aspera, L. limanda*, ***L. ferruginea***, *L. proboscideae, L. punctatissima, L. sakhalinensis*)
    - genus *Pseudopleuronectes* (***P. americanus***, *P. herzensteini, P. yokohamae*)
    - genus *Pleuronectes* (*P. glacialis, P. obscura, P. putnami, P. pinnifasciatus, P. platessa, P. quadrituberculatus*)
    - genus *Platichthys* (*P. bicoloratus, P. flesus, P. stellatus*)

Species selected for the molecular study are shown in bold face letters.

Figure 1A Relationship between the seven pleuronectid species involved in the molecular study extracted from the cladogram of Cooper (1996).

Figure 1B Relationship between the seven pleuronectid species involved in the molecular study extracted from the phenogram of Sakamoto (1984). Nomenclatural differences between Sakamoto (1984) and Cooper (1996) occur only at the genus level.


A
Limanda ferruginea
Pseudopleuronectes americanus
Microstomus zachirus
Microstomus pacificus
Isopsetta isolepis
Hippoglossoides elassodon
Reinhardtius hippoglossoides
B
Pleuronectes ferruginea
Pleuronectes americanus
Pleuronectes isolepis
Hippoglossoides elassodon
Errex zachirus
Microstomus pacificus
Reinhardtius hippoglossoides
90 %
98.99 % genus
similarity

Materials and Methods

Total DNA was isolated using standard methods (Maniatis et al. 1982) from muscle tissues of *Pseudopleuronectes americanus*, *Microstomus pacificus*, *Microstomus zachirus*, *Isopsetta isolepis*, *Hippoglossoides elassodon*, *Reinhardtius hippoglossoides*, *Limanda ferruginea* and, *Paralichthys dentatus*. Fish samples were kindly provided to us by the following institutes and individuals: Ted Pietsch, School of Fisheries, University of Washington, Seattle; Brian Urbain, School of Fisheries, University of Washington, Seattle; Ken Waiwood, St. Andrew's Biological Station, Department of Fisheries and Oceans Canada, St. Andrew's New Brunswick; Jean-Marie Sévigny, Maurice-Lamontagne Institute, Mont Joli, Québec and by Doug Chiperzak, Fisheries and Oceans Canada, Winnipeg, Manitoba.

A 1213 base pair portion of the COI gene was amplified using primers H7176 and L5950 (Table 2; Normark et al. 1991) by the polymerase chain reaction (PCR). PCR regimes consisted of two segments. The first segment consisted of 30 cycles of: i) denaturation at 95°C for 1 minute, ii) annealing at 43-47°C for 2 minutes and, iii) extension at 74°C for 2 minutes. The second segment consisted of a single cycle of extension at 74°C for 15 minutes.

Double stranded PCR products were cloned into a DH11S strain of *Escherichia coli* (Gibco BRL) using the SK$^+$/KS$^+$ pBluescript phagemid (Stratagene) cloning vectors. PCR products were prepared for ligation into the pBluescript plasmid by the Double GeneClean protocol (Bio101 Inc.) using DNA polymerase I (Pharmacia) and polynucleotide kinase

Table 2 PCR and sequencing primers used.

3' POSITION	Primer Sequence (5'-3')
H7176*	AGAAAATGTTGWGGGAARAA
H6868	TGIAGGTTGCSAGTCAGC
H6411	TGCTGTTGGTTTCATGTT
L5950*	ACAATCACAAAGAYATYGG
L6346	TCIGTAGACCTMACIATYTT
L6781	GTTTGRGCICAYCAYATGTT
T7	AATACGACTCACTATAG

H and L refer to the heavy and light strands of mitochondrial DNA.

Numbers indicate the position of the 3' end relative to the human mtDNA sequence (Anderson et al. 1981).

H7176 and L5950 (Normark et al. 1991) were used for PCR amplification, all other primers were used for sequencing only.

I = inosine, M=A/C, R=A/G, W=A/T, S=C/G, Y=C/T, K=G/T

(Pharmacia). The vector was prepared for ligation by incubation with the blunt-end cutting restriction enzyme, *Sma I* (Pharmacia). Three clones from each of the eight taxa being studied were sequenced in order to ensure sequence accuracy. Single stranded copies of each clone were sequenced by the dideoxy termination method (Sanger et al. 1977) using T7 DNA polymerase (Pharmacia) or Sequenase vs 2.0 (United States Biologicals) and sequencing primers (Table 2).

DNA sequences were manipulated using the GDE and SequEdit (Ell 1996) programs. An amino acid alignment, obtained using Clustal V (Higgins et al. 1991), was used as a template to align the DNA sequences for phylogenetic analysis.

Nucleotide composition was analyzed using the CODONS program of Lloyd and Sharp (1992). Compositional bias was analyzed using the chi-square method to test for a significant difference between the observed base frequency and the expected frequency of 25% at third codon positions of four fold degenerate codons.

The substitutional spectrum was analyzed by using parsimony criteria to trace the unambiguous sequence changes through the transversion parsimony tree using the MacClade program (Madison and Madison 1992). Unambiguous substitutions are sequence changes which have only one most parsimonious explanation through the tree.

Paralichthys dentatus (Paralichthyidae) was used as the outgroup in all phylogenetic analyses. A sister group relationship between Pleuronectidae and Paralichthyidae has been proposed based on jaw morphologies (Cooper 1996).

Parsimony analysis was carried out using PAUP version 3.1 (Swofford 1993). Trees were constructed by exhaustive search, with *P. dentatus* as an outgroup, using four different weighting schemes for transversions and transitions: i) equal weights, ii) transversions weighted 2 to 1 over transitions, iii) transversions weighted 3 to 1 over transitions and, iv) transversions only. A 100 replicate bootstrap analysis was preformed to test the stability of the nodes in each of the trees (Felsenstein 1988).

Distance analyses were carried out using the Phylip 3.5C package (Felsenstein 1991) and MEGA (Kumar et al. 1993). Genetic distances were calculated based on all substitutions (transition/transversion ratio of 3) and on transversions only using Kimura's two parameter model (Kimura 1980). Trees were constructed by the neighbour joining method in Phylip version 3.5C (Felsenstein 1991) and were rooted with *P. dentatus*. A 100 replicate bootstrap analysis was preformed to test the stability of the nodes in each of these trees (Felsenstein 1988). In order to ensure that the input order of taxa did not effect the final tree, neighbour joining analyses were repeated ten times with jumbled species input order for each replicate.

In order to determine whether there was an equal rate of evolution in all branches of the tree a relative rates test was performed. The relative rates test was done according to the method of Li and Tanimura (1987), using the number of synonymous and nonsynonymous substitutions with *P. dentatus* as the outgroup.

For a more detailed description of the materials and methods see appendix II.

Results

The alignment of a 1213 base pair portion of the COI gene from seven pleuronectid taxa and one outgroup (Appendix III) showed no evidence of insertions or deletions. This alignment is also available by anonymous FTP at bio01.bio.uottawa.ca in the /pub/COI directory. Most of the nucleotide sequence variation occurs at the third position of codons where 305 of the 315 substitutions are synonymous (Table 3). The first and second codon positions are highly conserved (Table 3). Twenty of the 30 substitutions occurring at the first codon position are synonymous changes between leucine codons, while all substitutions occurring at the second codon position are nonsynonymous. Analysis of unambiguous substitutions reveals a bias favouring transitions over transversions (Table 4). Transitions and transversions are also biased: 150 of the 228 transitions were in the form of C to T or T to C changes, while 60 of the 86 transversions involved changes between A and T or A and C (Table 4).

The overall nucleotide composition of the COI sequence (29.3% T, 27.9% C, 23.5% A and, 19.2% G) reflects the general asymmetric distribution of purines and pyrimidines between the light (L) and heavy (H) strands of animal mitochondrial DNA, and shows a bias against the occurrence of A and G on the light strand (Brown 1985). Because third codon positions of four fold degenerate codons are selectively neutral at the protein level the null hypothesis states that there will be an equal 25 % frequency of the four nucleotides at these sites. Chi squared tests reveal that the frequency of A is significantly higher than expected, while the frequency of G is significantly lower than expected at the third position of four fold

Table 3 The number and distribution of variable and parsimony informative sites in the alignment of COI sequences from eight flatfish species.

	Total	1st base of codons	2nd base of codons	3rd base of codons
Variable Sites	352	30	7	315
Parsimony informative sites	170	12	1	157

Table 4 Number of unambiguous transitions and transversions occurring between COI sequences of eight flatfish species. Values were obtained by tracing nucleotide changes through the transversion parsimony tree using the MacClade program of Madison and Madison (1992).

	to	A	C	G	T
from	A		20	55	21
	C	8		11	86
	G	23	6		3
	T	11	64	6	

Table 5 Percent nucleotide composition at the third position of four fold degenerate codons in the COI sequences of eight flatfish species. Chi squared tests were used to test the significance of the difference between the observed base frequencies and the expected base frequencies of 25%. Significant departure from a frequency of 25% was taken as an indication of a compositional bias.

	%T	%C	%A	%G
P. americanus	19.74	31.14	36.40**	12.72**
M. zachirus	20.44	34.67**	35.11**	09.7**
H. elassodon	23.25	30.26	33.77**	12.72**
R. hippoglossoides	25.11	31.28	34.36**	09.25**
L. ferruginea	21.59	31.28	37.00**	10.13**
M. pacificus	21.52	32.29*	35.87**	10.31**
I. isolepis	21.14	33.04*	35.24**	10.57**
P. dentatus	24.10	26.80	34.80**	14.30**

* indicates significant departure from 25 % composition (p< .05; df = 1)

**indicates significant departure from 25 % composition (p< .01; df = 1) degenerate codons thus revealing a preference for A and a bias against G at these sites (Table 5).

Phylogenetic trees produced by parsimony and distance methods based on all codon positions (Figures 2A, B) are congruent with the exception of the relationship between *M. pacificus* and *R. hippoglossoides*. Parsimony analysis returned one most parsimonious tree of 396 steps with a consistency index (c.i.) of 0.62 and the tree topology remained unchanged when transversions were weighted 2 to 1 and 3 to 1 over transitions. However, the bootstrap values tended to decrease with increasing weights for transversions (results not shown). Parsimony and distance analyses were repeated using only transversional substitutions in order to assess the effects of saturation of transitions on topology of the trees (Figure 3). These trees are congruent with one another, but not with the trees obtained from analyses of all substitutions with respect to the internal branching order of *P. americanus* and *L. ferruginea* (Figure 2).

Figure 2A Parsimony tree produced by exhaustive search option (PAUP 3.1) with equal weights for transitions and transversions and *P. dentatus* as the outgroup. The tree has a total of 396 steps and a consistency index of 0.62. Branch lengths are not proportional to the number of substitutions. Bold faced numbers at the nodes indicate percent bootstrap support; only values greater than 50% are shown. Numbers in parentheses represent the number of parsimony informative sites supporting that node.

Figure 2B Neighbour joining tree based on Kimura 2 parameter distances using *P. dentatus* as the outgroup. Pairwise distances were calculated using all codon positions and a transition/transversion ratio of 3. Numbers at the nodes indicate percent bootstrap support.

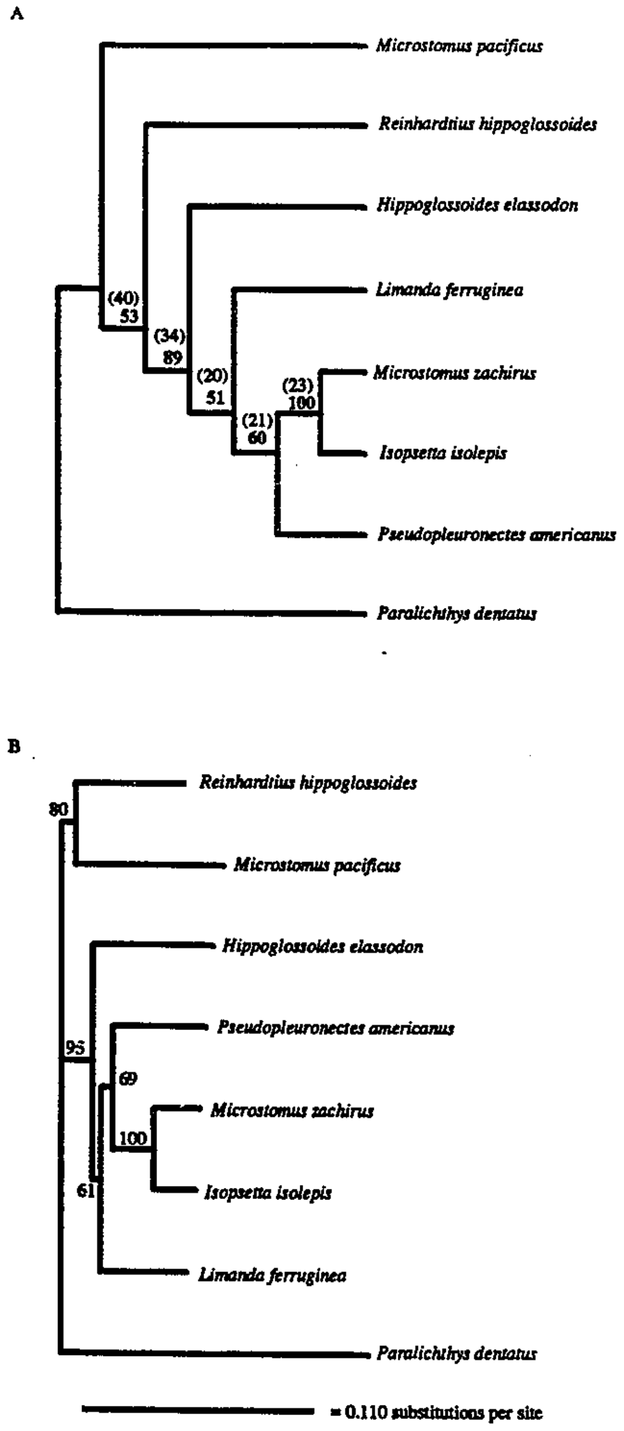
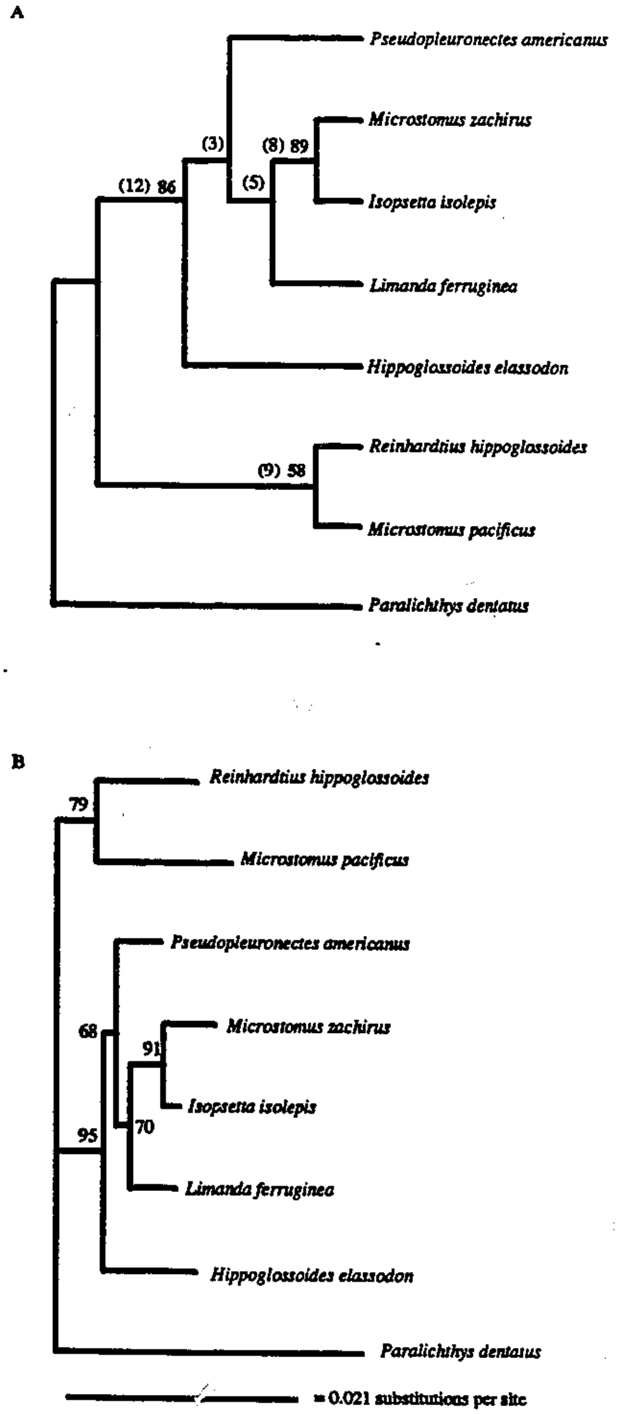

A
Microstomus pacificus
Reinhardtius hippoglossoides
Hippoglossoides elassodon
Limanda ferruginea
Microstomus zachirus
Isopsetta isolepis
Pseudopleuronectes americanus
Paralichthys dentatus
(40)
53
(34)
89
(20)
51
(21)
60
(23)
100
B
Reinhardtius hippoglossoides
Microstomus pacificus
Hippoglossoides elassodon
Pseudopleuronectes americanus
Microstomus zachirus
Isopsetta isolepis
Limanda ferruginea
Paralichthys dentatus
80
95
69
100
61
= 0.110 substitutions per site

Figure 3A Parsimony tree produced by exhaustive search of transversions only with *P. dentatus* as the outgroup. The tree has a total of 119 steps. Branch lengths are not proportional to the number of substitutions. Bold faced numbers at the nodes indicate percent bootstrap support; only values greater than 50% are shown. Numbers in parentheses represent the number of parsimony informative sites supporting that node.

Figure 3B Neighbour joining tree based on Kimura 2 parameter distances calculated with transversions only using *P. dentatus* as the outgroup. Numbers at the nodes indicate percent bootstrap support.


A
Pseudopleuronectes americanus
Microstomus zachirus
Isopsetta isolepis
Limanda ferruginea
Hippoglossoides elassodon
Reinhardtius hippoglossoides
Microstomus pacificus
Paralichthys dentatus
(3)
(8) 89
(12) 86
(5)
(9) 58
B
Reinhardtius hippoglossoides
Microstomus pacificus
Pseudopleuronectes americanus
Microstomus zachirus
Isopsetta isolepis
Limanda ferruginea
Hippoglossoides elassodon
Paralichthys dentatus
79
68
91
70
95
= 0.021 substitutions per site

Discussion

Analysis of our data shows that the COI gene has a conservative nature and that most of the substitutions occurring between species are synonymous. There is an apparent bias in the substitutional spectrum favouring transitions over transversions and a bias for the occurrence of particular transitions and transversions is also evident (Table 4). The overall nucleotide composition of the pleuronectid COI gene shows an asymmetric distribution of purines and pyrimidines between the light and heavy strands of mitochondrial DNA. Compositional analysis of neutral third codon positions shows a bias against G and in favour of A (Table 5). Phylogenetic analyses of the COI sequence based on different classes of substitutions produced competing hypotheses with respect to the relationship between *R. hippoglossoides* and *M. pacificus* and were unable to resolve the branching order of *L. ferruginea* and *P. americanus* (Figures 2 and 3).

Previous studies of animal mitochondrial genes and genomes (Brown et al. 1982; Brown and Simpson 1982; Desjardins and Morais 1989; Gadaleta et al. 1989; Arnason et al. 1991; Roe et al. 1985; Tzeng et al. 1992; Adkins and Honeycutt 1994; Brown et al. 1994) have shown that mitochondrial genes are characterized by a biased substitutional spectrum favouring transitions over transversions (Brown et al. 1982; Brown and Simpson 1982; Wolstenholme and Clary 1985; Brown et al. 1994) and strand specific nucleotide biases (Anderson et al. 1981; Brown and Simpson 1982; Adkins and Honeycutt 1994; Cantatore et al. 1994). Such characteristics are evident in our pleuronectid COI data. For instance there are 228 transitional substitutions compared to only 86 transversional substitutions (Table 4).

Furthermore, transitions involving C and T are almost twice as frequent as transitions involving A and G (Table 4). One possible explanation for the bias in the types of transitions that occur is that most of the substitutions occur at the third codon position where there is an observable nucleotide bias against G (Table 5). Therefore, there will be fewer G to A transitions relative to all other transitions (Table 4). This same explanation could be extended to transversions where changes from A to C or T are substantially more numerous than those from G to C or T (Table 4).

The overall nucleotide composition of the pleuronectid COI gene shows a strand specific bias against AG which is typical of animal mitochondrial DNAs (Brown et al. 1982). Each codon position exhibited a particular nucleotide bias (Table 6). The most plausible explanation for the biases at the first and second codon positions is that they are the result of biased amino acid usage (Adkins and Honeycutt 1994). The most commonly occurring amino acids in the COI protein are: leucine (approx. 13%), isoleucine (approx. 11%), glycine (approx. 11%), alanine (approx. 10%), valine (approx. 8%), phenylalanine (approx. 8%). Of these six amino acids valine, alanine, and glycine have codons with G at the first position and comprise approximately 30% of the codons in the COI sequence thus accounting for the high G content of the first codon position. Similarly codons for phenylalanine, leucine, isoleucine, and valine have T at the second position and comprise 40% of all codons in the COI sequence thus accounting for the high T content at this codon position. The bias against G at the third codon position is not as easily explained. If we look at the composition of the

Table 6 Percent nucleotide composition of codon positions in the COI sequence of eight flatfish species.

	1st position				2nd position				3rd position			
	T	C	A	G	T	C	A	G	T	C	A	G
P. americanus	17	23	27	33	44	26	16	14	26	34	28	12
M. zachirus	18	23	27	32	44	26	16	14	27	35	27	11
H. elassodon	17	23	27	32	44	26	16	14	26	36	27	11
R. hippoglossoides	17	23	27	32	44	26	17	14	29	35	27	09
L. ferruginea	17	23	27	33	44	26	17	13	26	36	29	09
M. pacificus	18	22	27	33	44	26	17	13	29	36	29	09
I. isolepis	18	23	27	33	44	26	16	14	26	35	27	11
P. dentatus	17	23	28	33	44	26	17	13	28	33	29	10

third codon position from fourfold degenerate codon families, where all bases are selectively neutral with regards to the gene product, the bias against G becomes even more apparent (Table 5). Such a bias against G at the third codon position in animal mitochondrial DNAs has been observed elsewhere (Adkins and Honeycutt 1994; Cantatore et al. 1994; Pumo et al. 1992; Kocher et al. 1989; Brown and Simpson 1982; Anderson et al. 1981). Two possible origins for the compositional bias have been proposed: 1) a biased mutational spectrum (Sueoka 1988) or, 2) selection at the nucleotide level (Sueoka 1988; Brown 1985) or perhaps a combination of these.

Results of phylogenetic analyses using different methods and categories of substitutions (transitions and transversions versus transversions only) produced competing hypotheses for the relationship between *R. hippoglossoides* and *M. pacificus,* as well as for the internal branching order of *P. americanus* and *L. ferruginea* (Figures 2 and 3). The fact that the bootstrap values are unsupportive (below 70%) of these nodes makes it difficult to choose between these competing hypotheses. In the case of the relationship between *R. hippoglossoides* and *M. pacificus,* it is the basal position of *M. pacificus* relative to all other pleuronectids which is of particular interest because this position for *M. pacificus* is largely incongruent with the morphological data. The fact that *M. pacificus* remains basal in all analyses provides confidence in this topology. Given that our analyses of the branching order of *P. americanus* and *L. ferruginea* were not congruent, this area of the tree is considered to be unresolved.

The fact that removal of the potentially saturated transitional substitutions did not improve bootstrap support of the nodes in these trees suggests that transitions are not phylogenetically misleading in this case. However, because the branch lengths leading to the outgroup (*P. dentatus*) in the distance trees indicate a large number of substitutions occurring between the outgroup and the ingroups, there is a potential that both transitions and transversions are saturated. To rule out this conclusion, we reanalyzed the data first using *R. hippoglossoides* and then *H. elassodon* as outgroups (see Appendix IV). The basal positions of *R. hippoglossoides* and *H. elassodon* with respect to the *P. americanus*/ *L. ferruginea* branches are consistently supported in all molecular analysis as well as in the morphology based cladogram (Cooper 1996) thus justifying their use as outgroups for analysis. Parsimony analyses based on all substitutions returned two most parsimonious trees with alternative branching orders for *P. americanus* and *L. ferruginea* when *R. hippoglossoides* and *H. elassodon* were used as outgroups (see appendix IV). Distance analysis with *R. hippoglossoides* as the outgroup produced a branching pattern identical to that of the transversional trees, and bootstrap values did not increase (see appendix IV). When *H. elassodon* was used as the outgroup distance analysis was unable to resolve the relationship between *P. americanus* and *L. ferruginea* (see Appendix IV). It is therefore unlikely that transversions are saturated.

Another potential problem for phylogenetic reconstruction is the occurrence of unequal rates of substitution in different lineages of the tree. Several of the more commonly used methods for phylogenetic reconstruction assume that the rate of nucleotide substitution is the same across all branches in the tree. If this assumption is violated then there is a possibility that phylogenetic reconstruction will be misled resulting in an erroneous tree. Relative rate tests using *P. dentatus* as the outgroup showed no significant variation in the rate of synonymous or nonsynonymous substitution across the tree (Table 7).

The molecular and morphology based cladograms are incongruent in several areas. The most striking conflict is the position of *M. pacificus.* In the morphology tree of Cooper (Figure 1A) *M. pacificus* and *M. zachirus* group together and are members of the Microstomini tribe within the Pleuronectinae (*sensu* Cooper 1996) occupying a terminal position in the tree. However, in the molecular trees (Figures 2 and 3), *M. pacificus* is basal to all other pleuronectid species, a position which suggests that the Pleuronectinae (*sensu* Cooper 1996) may not be monophyletic. For *M. pacificus* and *M. zachirus* to group together in the molecular tree would require an additional 29 steps in the tree and a drop in the consistency index from 0.62 to 0.56. It is interesting to note that in a phenetic study of the interrelationships of the Pleuronectidae (*sensu* Norman 1934) *M. pacificus* occupies a basal position (Figure 1B, Sakamoto 1984). Furthermore, although the position of *M. pacificus* in the morphology tree of Cooper (1996) is strongly supported by over 40 characters it is also characterized by 11 which would place it basal in the tree (Cooper, personal communication). However, due to the limited scope of the molecular study, the phenetic nature of Sakamoto's analysis (1984) and, the strong support in Cooper's morphological data, we feel that further corroborating evidence will be required before altering Cooper's (1996) classification.

Two possible explanations for the largely incongruent position of *M. pacificus* in the molecular tree relative to Cooper's (1996) morphology tree could be advanced.

First, the incongruence could be due to an error in the form of a misidentified fish or mislabelled genomic DNA or clone. If such an error occurred then the sequence which was

Table 7 Relative rate tests using the total number of synonymous and nonsynonymous substitutions with *P. dentatus* as outgroup.

	P. americanus	*M. zachirus*	*H. elassodon*	*R. hippoglossoides*	*L. ferruginea*	*M. pacificus*	*I. isolepis*
P. americanus	0.0000	0.2443 ± 0.2761	0.1673 ±0.2599	0.0053 ± 0.2314	0.0415 ± 0.2381	0.2684 ± 0.2815	0.0398 ± 0.2380
M. zachirus	0.0011 ± 0.0092	0.0000	0.0770 ± 0.2999	0.2496 ± 0.2756	0.2028 ± 0.2812	0.0241 ± 0.3188	0.2045 ± 0.2812
H. elassodon	0.0006 ± 0.0093	0.0005 ± 0.0092	0.0000	0.1726 ± 0.2594	0.1258 ± 0.2654	0.1011 ± 0.3050	0.1275 ± 0.2654
R. hippoglossoides	0.0011 ± 0.0092	0.0000 ± 0.0091	0.0005 ± 0.0092	0.0000	0.0468 ± 0.2376	0.2737 ± 0.2810	0.0451 ± 0.2375
L. ferruginea	0.0023 ± 0.0095	0.0034 ± 0.0094	0.0029 ± 0.0095	0.0034 ± 0.0094	0.0000	0.2269 ± 0.2865	0.0017 ± 0.2440
P. americanus	0.0000 ± 0.0093	0.0011 ± 0.0092	0.0006 ± 0.0093	0.0011 ± 0.0092	0.0023 ± 0.0095	0.0000	0.2286 ± 0.2865
I. isolepis	0.0006 ± 0.0093	0.0005 ± 0.0092	0.0000 ± 0.0092	0.0005 ± 0.0092	0.0029 ± 0.0095	0.0006 ± 0.0093	0.0000

Relative rate scores based on synonymous substitutions and standard errors are shown in the top right of the matrix.

Relative rate scores based on nonsynonymous substitutions and standard errors are shown in the bottom left of the matrix.

None of the relative rate differences are significantly different from zero at the 5% level.

used to represent *M. pacificus* in the phylogenetic analysis may not have come from *M. pacificus*. To rule out these possibilities the identification of *M. pacificus* was confirmed using the key of Norman (1934) and a PCR test was performed. COI sequence amplified from new *M. pacificus* genomic DNA stock was digested with restriction enzymes. The restriction profile obtained from the newly amplified COI sequence matched the expected profile obtained by computer analysis of the existing *M. pacificus* COI sequence, thus ruling out the possibility of misidentification or mislabelling.

Second, there is the possibility of mitochondrial introgression. Mitochondrial introgression is a process whereby through hybridizations between closely related species whose ranges overlap the mitochondrial genome of one species is passed on to the second and eventually becomes fixed in that population (Avise 1994). Chow and Kishino (1995) state that mitochondrial introgression between fish species does not seem to be unusual and, furthermore, hybrid flatfishes have been identified (ie. *Inopsetta ischyra*, Hart 1973). If *M. pacificus* in our study has come from a population where mitochondrial introgression has occurred and if the source of the introgressed mitochondrial genome is a basal pleuronectid or even a non-pleuronectid flatfish then the basal position of *M. pacificus* would be explained. If such an event has occurred, then trees produced using other mitochondrial genes should give the same topology as COI and all mitochondrial trees should be incongruent with trees produced from nuclear sequences.

A further point of incongruence between morphology and molecular trees is the position of *P. americanus* and *L. ferruginea*. However, the instability of the molecular tree in this area does not allow for a comparison. The extremely short branch lengths separating *L.*

ferruginea and *P. americanus* in the distance trees (Figures 2B and 3B) indicate that there is very little genetic variability between the COI genes of these two species. This lack of sequence variation is also evident in the transversional parsimony tree where there are very few characters which support the branching order for *P. americanus* and *L. ferruginea.* It is interesting to note that the lack of overall, and parsimony informative variation in this area of the molecular trees is mirrored by a similar shortage of characters in the morphological data (Cooper, personal communication). On the other hand, there seems to be a sufficient number of characters supporting the tree topology obtained by parsimony analysis of all classes of substitutions (Figure 2A). However, the low bootstrap values at nodes which are supported by 20 and 21 characters (Figure 2A) indicate a high degree of homoplasy in the data. Despite the incongruences between the data sets, this study has added support for the relative positions of the pleuronectid subfamilies (Hippoglossinae, Hippoglossoidinae and, Pleuronectinae) in the newly proposed morphological classification of Cooper (Table 1).

Conclusion

The purpose of the molecular study was to test a newly proposed hypothesis for pleuronectid classification (Cooper 1996) using an independent data set. The molecular data calls into question the status of the subfamily Pleuronectinae (*sensu* Cooper 1996) and the interrelationships of certain species of Pleuronectinae remain in question due to homoplasy and a lack of characters supporting one particular topology for some internal nodes of the molecular trees. The lack of characters from independent data sets may indicate that nodes in this area of the tree arose within a very small time span. If this were true, there would be very few data sets which could fully resolve this area of the tree. However, we cannot rule out the possibility that COI, because of its conserved nature, is not the ideal phylogenetic marker in this case. Hillis and Moritz (1990) state that expectations that one gene will be ideal for recovering all phylogenetic relations within a given group of organisms is perhaps not realistic and propose use of sequences from several different genes to resolve different regions in a tree. The COI data have provided strong support for the proposed relationship of the pleuronectid subfamilies as well they have indicated that resolution of pleuronectin relationships will require sequences that evolve much faster than COI , and/or sampling a larger number of pleuronectin species.

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Appendix I: Taxonomic History of the Right-Eyed Flounders (Family: **Pleuronectidae**)

Jordan and Evermann (1898) placed all flatfishes into the suborder Heterosomata and divided it into two families: **Pleuronectidae** and Soleidae. The **Pleuronectidae** (*sensu* Jordan and Evermann 1889) were defined by: a preopercular margin more or less distinct which was not hidden by the skin and scales of the head, large eyes which were well separated, a moderate to large sized mouth and, the presence of teeth. Jordan and Evermann (1898) divided the **Pleuronectidae**, which contained nearly 500 species, into 6 subfamilies: Pleuronectinae, Samarinae, Hippoglossinae, Psettinae, Oncopterinae and, Pelecanichthyinae.

In Regan's (1910) classification all flatfishes were placed into the order Heterosomata which was divided into three new suborders: Psettodoidae, Pleuronectoidea and, Soleoidea. Within the Pleuronectoidea, Regan (1910) defined 3 families: Bothidae, Paralichthoididae and, **Pleuronectidae**. The **Pleuronectidae** (*sensu* Regan 1910) were divided into three subfamilies: Pleuronectinae, Samarinae and, Rhombosoloeinae and were characterized as being right-eyed and having no oil globules present in the egg. Regan (1929) revised this classification by omitting the suborders in his original classification and recognizing 5 families: Psettodidae, Bothidae, **Pleuronectidae**, Soleidae, and Cynoglossidae. Norman (1934) agreed with Regan's classification but added one subfamily, the Poecilopsettinae, to the **Pleuronectidae**. Thus, the classification of the flatfishes (*sensu* Norman 1934) was:

- Order Heterosomata
  - Suborder Psettodoidea
    - Family Psettodidae
  - Suborder Pleuronectoidea
    - Family Cynoglossidae
    - Family Soleidae
    - Family **Pleuronectidae**
      - Subfamily Pleuronectinae
      - Subfamily Poecilopsettinae
      - Subfamily Samarinae
      - Subfamily Paralichthodinae
      - Subfamily Rhombosoleinae
    - Family Bothidae
      - Subfamily Paralichthinae
      - Subfamily Bothinae
      - Subfamily Scophthalminae

Hubbs (1945) altered slightly Regan's (1910) and Norman's (1934) classification of the **Pleuronectidae** by removing two genera: *Brachypleura* and *Lepidoblepharon* from the Samarinae and placing them in the family Citharidae which he regarded as a member of the suborder Pleuronectoidea.

This classification of the **Pleuronectidae** (Norman 1934) with the revisions of Hubbs

(1945) remained unchallenged until Hensely and Ahlstrom (1984) the latter questioned the monophyletic status of the **Pleuronectidae** (*sensu* Regan and Norman) on the basis of the plesiomorphic states of characters used to define the group. Further cladistic studies of flatfish interrelationships (Chapleau and Keast 1988) concurred with Hensley and Ahlstrom (1984) with regards to the dubious nature of the monophyletic status of the **Pleuronectidae** (*sensu* Regan and Norman). In a study of the soles (Soleidae), Chapleau and Keast (1988) made a preliminary recommendation that the paraphyletic **Pleuronectidae** of Regan (1910) and Norman (1934) be dismembered by elevating its subfamilies to the family level provided that there was sufficient evidence to support a monophyletic status for each of the subfamilies (*sensu* Norman). Thus, the Pleuronectinae (*sensu* Norman 1934) became the **Pleuronectidae** *sensu* Chapleau and Keast (1988). However, a cladistic reassessment of the Pleuronectiformes (Chapleau 1993) found that most of the characters traditionally used to define the pleuronectid subfamilies (*sensu* Regan and Norman) were unreliable and thus the monophyletic status of the **Pleuronectidae** (*sensu* Chapleau and Keast 1988) was not supported. In a cladistic study of 53 flatfishes from the family **Pleuronectidae** (*sensu* Chapleau and Keast 1988) Cooper (1996) found 10 apomorphies uniting the **Pleuronectidae** (*sensu* Chapleau and Keast 1988) as a monophyletic group. The result of Cooper's cladistic analysis is a new classification (Figure I) for the **Pleuronectidae** (*sensu* Chapleau and Keast 1988).

Appendix II: Materials and Methods

DNA isolation

Total DNA was isolated from *Pseudopleuronectes americanus, Microstomus pacificus, Microstomus zachirus, Isopsetta isolepis, Hippoglossoides elassodon, Reinhardtius hippoglossoides, Limanda ferruginea* and, *Paralichthys dentatus* using a method adapted from Maniatis et al. (1982). Approximately 4 grams of muscle tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle and were dissolved in approximately 10 volumes of extraction buffer (10 mM tris HCl pH 8.0, 0.1M EDTA pH 8.0, 20 mg/ml pancreatic RNase A, 0.5% SDS and, 100 μg/ml proteinase K). The extraction mixture was incubated at 65°C for 3 hours and was then phenol extracted three times with centrifugation at 4500 rpm for 15 minutes. The supernatant was chloroform:isoamyl alcohol (24:1) extracted twice. DNA was precipitated by addition of 0.2 volumes of 10 M ammonium acetate (NH_3OAc) and two volumes of cold 95% ethanol and centrifugation at 4500 rpm for 20 minutes. The DNA was then washed twice with 70% ethanol and resuspended in TE (pH 8.0).

PCR Amplification

COI sequences were amplified with degenerate primers H7176 and L5950 (Table 2, Normark et al. 1991) by the polymerase chain reaction. These primers were known to target an approximate 1226 base pair portion of the COI gene. PCR was carried out by combining approximately 150 ng of DNA, 40 pmol each of H7176 and L5950, 10μL of 10x Taq thermo buffer (Promega), 8μL of 2.5 mM deoxynucleotide triphosphate (dNTP) mixture, 8-12 μL of 25 mM $MgCl_2$, 0.5 units of Taq polymerase (Promega) and, distilled water to a final volume of 100 μL. The PCR mixture was boiled for 5 minutes and then quenched on ice for 5 minutes before the Taq polymerase was added. After centrifugation for 10 seconds each reaction was layered with paraffin oil to prevent condensation during thermal cycling. The PCR regime consisted of two programs the first had three steps: 1) denaturation for one minute at 95°C, 2) annealing for two minutes at 43-47°C, 3) extension for two minutes at 74°C. Thirty cycles of program one were completed before passing to the second program which consisted of one extension step of 15 minutes at 74°C. PCR products were extracted with an equal volume of phenol:chloroform (1:1) and 10 μL of each reaction was run on an 0.8% agarose gel with 1x TBE running buffer. PCR products were visualized by staining with ethidium bromide and viewed using ultraviolet light.

Cloning of PCR Products

PCR products were prepared for blunt-end cloning using the double GeneClean protocol (Bio 101 Inc.). PCR products were run on an 0.8% agarose gel with 1x TAE running buffer and bands corresponding in size to the COI fragment were excised. DNA was purified from agarose using the GeneClean protocol (Bio 101 Inc.) and resuspended in 80 μL of TE. The PCR DNA was then made blunt-ended by incubation with 10 μL of 10x Pol I buffer (0.5 M tris pH 7.5, 0.1M $MgCl_2$, 10mM DTT, 0.5mg/ml BSA and, 200 uM dNTPs), 10 μL of 10 mM ATP, and 10 units each of polynucleotide kinase (Pharmacia) and T4 DNA polymerase I (Pharmacia) for one hour at 37°C. The reaction was terminated by addition of

1μL of 0.5 M EDTA, DNA was recovered using the GeneClean protocol (Bio 101 Inc.).

The pBluescript SK^+/KS^+ phagemid cloning vectors (Stratagene) were used to clone the PCR products into a strain of DH11S *E. coli* (Gibco). The pBluescript plasmids have several features which were exploited in this study. Presence of an ampicillin resistance gene allows for selection of transformed cells on medium containing ampicillin, a polycloning site within the *lacZ* gene allows for blue/white selection of recombinant colonies when cells are grown on medium containing IPTG and Xgal and, an origin of replication for the F1 filamentous phage allows single strand copies of the plasmid to be generated for single strand sequencing. The pBluescript plasmid comes in two versions SK^+ and KS^+, these differ by having opposite orientations of the origin of F1 replication relative to the polycloning site, making it possible to obtain single stranded DNA for both strands of a clone.

The KS^+ version of the pBluescript plasmid was prepared for blunt-end ligation with PCR products by digestion with *Sma* I. This reaction was carried out in a 100 μL volume containing 10 μg of vector DNA, 10 μL of 10x one-phor-all buffer (Pharmacia) and 50 units of *Sma* I (Pharmacia) the reaction was terminated by incubation at 65°C for 5 minutes. Digested plasmid DNA was then recovered by electrophoresis on a 0.8% agarose gel with 1x TAE running buffer and purification by GeneClean (Bio 101 Inc.). The digested plasmid DNA was next dephosphorylated by incubation with 10 μL of 10x calf intestine alkaline phosphatase (CIP) buffer (Boehringer Mannheim) and 3 units of alkaline phosphatase (Boehringer Mannheim) in a total volume of 100 uL. This mixture was incubated at 56°C for 15 minutes at which time an additional 3 units of alkaline phosphatase were added to the reaction, this mixture was further incubated at 37°C for 45 minutes. The reaction was halted by the addition of 2 μL of 0.5 M EGTA and incubation at 65°C for 15 minutes. Digested dephosphorylated plasmid DNA was then purified using the GeneClean protocol (Bio 101 Inc.).

Blunt-ended PCR products were ligated into the *Sma* I polycloning site of the KS$^+$ pBluescript plasmid. Ligation reactions were carried out in a 10 μL total volume containing 5.5 μL of PCR DNA, 1μL of digested dephosphorylated plasmid DNA, 1 μL of 10x one phor-all buffer (Pharmacia), 0.5 μL 10 mM ATP, 1 μL 10 mM hexamine cobalt chloride and, 8 units of T4 ligase (Pharmacia). Reactions were incubated at 10°C for 8 hours. The ligase was inactivated by a five minute incubation at 65°C.

Transformation into **E. coli**

Ligation products were transformed into a DH11S strain of *E. coli* (Gibco) competent cells. Five μL of the ligation reaction were diluted 1:1 with distilled water and this mixture was added to 100 μL of DH11S competent cells. The cells were then incubated on ice for 1 hour before being heat shocked for 2 minutes at 37°C. One ml of LB medium was added to the transformation and the cells were allowed to grow for one hour at 37°C. After 1 hour cells were spun down for 30 seconds at 14 000 rpm, the cells were then resuspended in 100 μL of LB medium and were plated on solid LB medium (1.5% agarose) containing 50 μg/ml of ampicillin, 0.5 uM IPTG (Isopropylthiogalactoside) and, 0.01% X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside).

IPTG acts as an inducer of the *lacZ* gene present on the plasmid. The polycloning site of the plasmid lies within the *lacZ* gene thus any insertion into the cloning site will disrupt the reading frame of the *lacZ* gene rendering it non functional. The *lacZ* gene product, ß-galactosidase, metabolizes lactose but cannot metabolize IPTG. Xgal is a chromogenic analog of lactose which, when metabolized by ß-galactosidase activity causes colonies to appear blue in colour. The *lacZ* reading frame of nonrecombinant plasmids will not be disrupted by inserts in the polycloning site and will have ß-galactosidase capability when induced and these colonies will be blue in colour. Recombinant plasmids will have inserts in the polycloning site disrupting the *lacZ* reading frame rendering their *lacZ* genes nonfunctional, and these colonies will appear white in colour in the presence of IPTG and X-gal.

Screening for clones

White colonies were screened for the presence of COI inserts by the alkali mini-prep method described in Maniatis et al. (1982). White colonies were picked from the solid medium using sterile tooth picks and were grown overnight at 37°C in 3 ml of LB medium containing 50 µg/ml ampicillin, 1.5 ml of the overnight culture was placed into a 1.5 ml Eppendorf tube, centrifuged for 30 seconds at 14 000 rpm, the supernatant was discarded, and the cell pellet was resuspended in 100 µL of solution I (50 mM glucose, 50 mM tris HCl pH 8.0 and 50 mM EDTA pH 8.0). Cells were then lysed by addition of 200µL of freshly prepared solution II (0.2 M NaOH and 1% SDS) and incubation on ice for 15 minutes. Cellular remnants were precipitated by addition of 150 µL of solution III (5 M KOAc, 1.5 M glacial acetic acid, 28.5 ml of distilled water), incubation on ice for 15 minutes, and centrifugation at 14 000 rpm for 10 minutes. The supernatant was extracted with an equal volume of phenol:chloroform (1:1), and plasmid DNA precipitated with 2 volumes of cold

95% ethanol and centrifugation for 15 minutes at 14 000 rpm. The DNA was then washed once with 500 µL of 70% ethanol and resuspended in 100 µL of TE pH 8.0 containing 20 µg/ml DNase free pancreatic RNase A(Pharmacia). After incubation at 37°C for 15 minutes 2 µL of mini-prep DNA was digested with the restriction enzymes *Eco* R1 (Pharmacia) and *Bam* H1 (Pharmacia) to verify the presence and size of the insert.

Preparation of competent cells

DH11S *E. coli* cells were made competent using the rubidium chloride method of Hanahan (1985). Two ml of LB medium were inoculated using a single colony and grown overnight at 37°C with vigorous shaking. The overnight culture was used to inoculate 400 ml of LB medium (containing 20 mM $MgSO_4$) this culture was grown at 37°C for 3 hours, cells were then pelleted by centrifugation at 4500 g for 5 minutes. The cell pellet was resuspended in 40 ml of cold TFB1(30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, and acetic acid to pH 5.8) and incubated on ice for 10 minutes. Cells were then pelleted by centrifugation at 5000 rpm for 5 minutes and resuspended in 10 ml of cold TFB2 (10 mM PIPES, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol and, KOH to pH 6.5). After 60 minutes of incubation on ice the cells were aliquoted into 100 µL portions and were quick-frozen in an ethanol/dry ice bath and stored at -70°C.

Preparation of single stranded DNA

Single strand DNA for sequencing was prepared by inoculating 2 ml of LB medium (containing 50 µg/ml ampicillin and 10 mM $MgCl_2$) and incubation at 37°C. Following an initial 3-4 hours of incubation 10 µL of the M13K07 helper phage was added to the culture and the volume was brought to 10 ml by addition of LB medium (containing 50 µg/ml ampicillin and 10 mM $MgCl_2$). The 10 ml culture was grown overnight with vigorous shaking. The cells were precipitated by centrifugation at 8000 rpm for 15 minutes and the viral particles which remained in the supernatant were precipitated by addition of 2.5 ml 20% polyethylene glycol (mw 2000) containing 2.5 M NaCl and incubation on ice for one hour. Viral particles were pelleted by centrifugation at 8000 rpm for 15 minutes and the supernatant was discarded. The viral pellet was resuspended in 800 µL of TE pH 8.0 containing 0.5% SDS, 2.5 µg/ml proteinase K and, 40 µg/ml of RNase A and incubated at 65°C for 2 hours. Viral remnants were precipitated by addition of 5 M potassium acetate and centrifugation at 14 000 rpm for 10 minutes, the supernatant was extracted with an equal volume of phenol:chloroform (1:1).. Single stranded DNA was precipitated from the supernatant by addition of 3 volumes of cold 95% ethanol and centrifugation at 14 000 rpm for 15 minutes. Following precipitation the single stranded DNA pellet was washed with 70% ethanol, resuspended in 60 µL of TE (pH 8.0) and, visualized on an agarose gel by ethidium bromide staining.

DNA Sequencing

Single stranded copies of KS^+ clones were sequenced by the dideoxy method (Sanger et al. 1977) using the T7 sequencing kit (Pharmacia) and T7 DNA polymerase (Pharmacia) or Sequenase vs 2.0 (United States Biologicals, Cleveland OH.). After determining that these initial sequences corresponded to the COI gene, the inserts were cloned into the SK^+ version of pBluescript so that both strands could be sequenced. Because of the apparent error rate of the Taq polymerase enzyme, 3 different clones from each taxon were sequenced to ensure sequence accuracy. An initial 400 base pairs of each clone were sequenced using the T7 primer located on the KS^+ and SK^+ plasmids, these sequences were aligned so that additional internal primers (Table 2) could be designed for further sequencing.

DNA sequences were entered into an IBM clone computer from autoradiograms using the program DNA Parrot DP 100-PC version 2.3. The DNA sequences were then manipulated using the sequence editing programs: GDE, running on a SUN workstation, and Sequedit (Ell 1996), running on a PC. The DNA sequences were aligned manually using GDE.

Appendix III: Alignment of Pleuronectid COI DNA Sequences.

P. dentatus	CACCCTCTAT	CTCATATTTG	GTGCCTGAGC	CGGAATAGTG	GGAACAGCCC
P. americanus	CACCCTCTAT	CTTGTATTTG	GTGCCTGAGC	CGGAATAGTG	GGGACGGGCC
E. zachirus	CACCCTCTAT	CTCGTATTTG	GTGCCTGAGC	CGGAATAGTG	GGGACAGGCT
H. elassodon	CACCCTCTAT	CTCGTATTTG	GTGCCTGAGC	CGGAATAGTG	GGGACAGGCC
R. hippoglossoides	CACCCTCTAT	CTCGTATTTG	GTGCCTGAGC	CGGTATAGTG	GGAACAGGCC
L. ferruginea	CACCCTCTAT	CTCGTATTTG	GTGCCTGAGC	CGGAATAGTG	GGGACAGGCC
M. pacificus	CACCCTCTAT	CTCGTATTTG	GTGCCTGAGC	CGGAATAGTG	GGCACAGGCC
I. isolepis	CACCCTCTAT	CTCGTATTTG	GTGCCTGAGC	CGGAATAGTG	GGGACAGGCT

P. dentatus	TGAGTTTACT	CATTCGGGCA	GAACTTAGCC	AACCCGGCGC	CCTGCTAGGA
P. americanus	TAAGTCTGCT	CATTCGAGCA	GAGCTAAGCC	AACCCGGGGC	TCTCCTGGGA
E. zachirus	TAAGTCTGCT	TATTCGAGCA	GAGCTTAGCC	AACCCGGGGC	TCTCCTGGGA
H. elassodon	TAAGTCTGCT	CATTCGAGCA	GAACTCAGCC	AACCTGGGGC	TCTCCTGGGA
R. hippoglossoides	TAAGTCTGCT	TATTCGGGCA	GAACTAAGCC	AACCTGGGGC	TCTCCTTGGA
L. ferruginea	TAAGTCTGCT	CATTCGAGCA	GAACTAAGCC	AACCTGGGGC	TCTCCTGGGA
M. pacificus	TAAGTCTGCT	TATTCGAGCA	GAACTAAGCC	AACCTGGGGC	TCTCCTAGGA
I. isolepis	TAAGTCTACT	CATTCGGGCA	GAACTTAGCC	AACCTGGGGC	TCTCCTGGGA

P. dentatus	GACGACCAGA	TTTATAACGT	AATCGTTACT	GCACACGCCT	TTGTAATAAT
P. americanus	GACGACCAAA	TTTATAATGT	AATCGTCACC	GCACACGCCT	TTGTAATAAT
E. zachirus	GACGACCAAA	TTTATAACGT	AATCGTCACC	GCACACGCCT	TTGTAATAAT
H. elassodon	GACGATCAAA	TTTATAACGT	GATCGTTACC	GCACACGCCT	TTGTAATAAT
R. hippoglossoides	GACGACCAAA	TTTATAACGT	AATCGTCACC	GCACACGCCT	TTGTAATAAT
L. ferruginea	GACGACCAAA	TTTATAACGT	AATCGTCACC	GCACACGCCT	TTGTAATAAT
M. pacificus	GACGACCAAA	TTTATAACGT	AATCGTCACC	GCACACGCCT	TTGTAATAAT
I. isolepis	GACGACCAAA	TTTATAACGT	AATCGTCACC	GCACACGCCT	TTGTAATAAT

P. dentatus	CTTTTTTATG	GTAATACCAA	TTATAATCGG	AGGGTTTGGG	AACTGGCTTA
P. americanus	CTTCTTTATA	GTAATACCAA	TTATGATCGG	AGGGTTCGGA	AACTGACTTA
E. zachirus	CTTTTTTATA	GTAATGCCAA	TTATGATCGG	AGGGTTCGGA	AACTGACTTA
H. elassodon	CTTCTTTATA	GTAATACCAA	TTATGATCGG	AGGGTTCGGA	AACTGACTTA
R. hippoglossoides	CTTTTTTATA	GTAATACCCA	TTATGATCGG	GGGTTTCGGA	AACTGGCTTA
L. ferruginea	TTTCTTTATA	GTAATGCCAA	TTATGATTGG	AGGGTTCGGA	AACTGACTTA
M. pacificus	CTTTTTTATA	GTAATACCCA	TTATGATTGG	GGGCTTTGGA	AACTGACTTA
I. isolepis	CTTCTTTATA	GTAATGCCAA	TTATGATCGG	AGGGTTCGGA	AACTGACTTA

P. dentatus	TTCCCCTCAT	AATTGGTGCC	CCAGATATAG	CATTTCCTCG	AATAAACAAT
P. americanus	TTCCATTAAT	AATTGGGGCC	CCCGATATGG	CCTTCCCTCG	AATAAATAAC
E. zachirus	TTCCATTAAT	AATTGGGGCC	CCCGATATGG	CCTTCCCTCG	AATAAATAAT
H. elassodon	TCCCGCTAAT	GATCGGAGCC	CCCGATATGG	CTTTCCCTCG	GATAAATAAC
R. hippoglossoides	TTCCACTAAT	AATTGGAGCC	CCAGATATGG	CTTTCCCTCG	AATAAATAAC
L. ferruginea	TCCCATTAAT	AATTGGGGCC	CCCGATATGG	CCTTCCCTCG	AATAAACAAC
M. pacificus	TCCCCTTAAT	AATTGGGGCC	CCTGACATAG	CGTTCCCCCG	AATAAATAAC
I. isolepis	TTCCATTAAT	AATTGGGGCC	CCCGATATGG	CCTTCCCTCG	AATGAACAAC

P. dentatus	ATAAGCTTTT	GACTATTACC	TCCTTCATTC	CTTCTTCTTT	TAGCTTCCTC
P. americanus	ATGAGTTTCT	GACTCCTACC	CCCATCCTTT	CTCCTCCTTC	TAGCCTCTTC
E. zachirus	ATGAGTTTCT	GACTTCTACC	CCCATCCTTT	CTCCTCCTTC	TAGCCTCTTC
H. elassodon	ATGAGCTTCT	GACTTCTACC	CCCATCGTTT	CTTCTCCTCC	TAGCCTCTTC
R. hippoglossoides	ATGAGTTTCT	GACTTCTTCC	CCCATCCTTT	CTTCTCCTCT	TAGCCTCTTC
L. ferruginea	ATGAGTTTCT	GACTTCTTCC	CCCATCCTTT	CTCCTTCTCC	TAGCCTCTTC
M. pacificus	ATGAGTTTCT	GACTCCTTCC	CCCATCCTTT	CTGCTACTCC	TAGCCTCTTC
I. isolepis	ATGAGTTTCT	GACTTCTACC	CCCGTCCTTT	CTCCTTCTTC	TAGCCTCTTC

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P. dentatus          GGGTGTTGAA GCTGGTGCCG GTACCGGATG AACTGTCTAC CCTCCTTTAG
P. americanus        AGGCGTCGAA GCTGGGGCAG GGACAGGATG AACCGTGTAT CCCCCACTAG
E. zachirus          AGGCGTTGAA GCTGGGGCAG GAACAGGATG AACCGTTTAT CCCCCGTTAG
H. elassodon         AGGTGTAGAA GCCGGGGCTG GGACAGGATG AACCGTATAT CCTCCCCTGG
R. hippoglossoides   AGGTGTTGAA GCTGGGGCAG GTACGGGGTG AACCGTTTAT CCACCACTAG
L. ferruginea        AGGTGTTGAA GCCGGGGCGG GAACAGGTTG AACCGTTTAC CCCCCATTAG
M. pacificus         AGGTGTTGAA GCAGGGGCAG GTACTGGGTG AACCGTGTAC CCCCCACTAG
I. isolepis          AGGCGTTGAA GCTGGGGCAG GAACAGGATG AACCGTATAT CCCCCGTTAG


P. dentatus          CAGGCAACCT GGCTCATGCC GGAGCCTCGG TAGATCTAAC TATCTTTTCA
P. americanus        CTGGAAATCT AGCACACGCC GGAGCATCAG TAGACCTCAC CATTTTCTCC
E. zachirus          CTGGAAATCT AGCACACGCC GGAGCCTCCG TAGACCTCAC AATCTTCTCT
H. elassodon         CTGGAAATCT GGCACACGCC GGAGCGTCCG TAGACCTCAC AATCTTCTCT
R. hippoglossoides   CTGGTAATCT GGCCCACGCC GGAGCATCCG TTGACCTAAC AATCTTCTCA
L. ferruginea        CTGGGAACCT AGCACATGCC GGGGCATCCG TAGACCTCAC AATCTTCTCT
M. pacificus         CTGGCAATCT AGCCCATGCT GGAGCATCTG TAGACCTAAC AATCTTCTCA
I. isolepis          CTGGAAATCT AGCACACGCC GGAGCATCAG TAGACCTCAC AATCTTCTCT

P. dentatus          CTTCACCTTG CAGTTATCTC GTCAATTCTA GGAGCTATCA ACTTCATTAC
P. americanus        CTCCACCTTG CCGGAATTTC ATCAATTCTA GGGGCAATCA ACTTTATTAC
E. zachirus          CTTCACCTCG CCGGAATTTC ATCAATTCTG GGGGCCATCA ACTTTATTAC
H. elassodon         CTTCACCTTG CCGGAATTTC ATCAATCCTG GGAGCAATCA ACTTTATTAC
R. hippoglossoides   CTTCACCTTG CAGGAATTTC GTCAATTCTG GGGGCAATTA ACTTTATTAC
L. ferruginea        CTTCACCTTG CCGGAATTTC ATCAATTCTG GGGGCAATCA ACTTTATTAC
M. pacificus         CTACACCTCG CAGGTATCTC CTCAATTCTG GGAGCTATCA ACTTTATCAC
I. isolepis          CTTCACCTTG CCGGAATTTC ATCAATTCTG GGGGCCATCA ACTTTATTAC

P. dentatus          TACCATCATA AACAACGACC CGACAGCGGT CACTATATAC CAAATACCCC
P. americanus        TACTATCATC AACATGAAAC CAACAGCAGT CACTATGTAC CAAATCCCAC
E. zachirus          TACCATCATC AACATGAAAC CGACAGCAGT CACTATGTAT CAAATCCCAC
H. elassodon         CACCATCATC AACATGAAAC CTACAGCGGT CACTATATAC CAAATCCCAC
R. hippoglossoides   TACCATCATC AACATGAACC CAACAACAGT TACTATGTAC CAAATCCCAT
L. ferruginea        TACCATCATC AATATGAAAC CAACAGCAGT CACTATGTAC CAAATCCCAC
M. pacificus         CACCATTATT AACATGAAAC CTGCAACAGT GACCATGTAC CAAATCCCAT
I. isolepis          TACCATCATC AACATGAAAC CGACAGCAGT CACTATGTAC CAAATTCCCC

P. dentatus          TATTTATTTG GGCCGTACTG ATTACAGCTG TCCTCTTACT CCTCTCTCTG
P. americanus        TATTTGTCTG GGCCGTACTA ATCACTGCCG TCCTTCTTCT TCTTTCCCTA
E. zachirus          TGTTTGTCTG AGCCGTACTA ATCACTGCCG TTCTTCTCCT CCTTTCTCTA
H. elassodon         TATTTGTGTG AGCCGTACTA ATCACGGCCG TTCTTCTCCT CCTTTCCCTT
R. hippoglossoides   TATTTGTTTG AGCCGTTCTA ATTACCGCCG ATCTTCTTCT TCTGTCCCTT
L. ferruginea        TATTTGTTTG AGCCGTACTA ATTACCGCCG TTCTTCTTCT CCTTTCCCTT
M. pacificus         TATTTGTCTG AGCTGTTTTA ATTACCGCTG TCCTCCTCCT CCTATCACTT
I. isolepis          TGTTTGTCTG AGCCGTTCTA ATCACTGCCG TTCTTCTCCT CCTTTCTCTA

P. dentatus          CCAGTCCTAG CCGCTGGCAT TACAATACTG CTGACGGACC GAAACCTGAA
P. americanus        CCCGTCCTGG CCGCTGGCAT TACAATGCTA CTGACAGACC GCAACCTAAA
E. zachirus          CCCGTCTTAG CCGCTGGAAT TACAATGCTA CTTACAGACC GCAACCTAAA
H. elassodon         CCAGTCTTAG CGGCTGGCAT CACAATGCTA TTAACAGACC GCAACCTAAA
R. hippoglossoides   CCCGTCTTAG CCGCAGGGAT TACAATGCTA CTAACAGACC GCAACCTCAA
L. ferruginea        CCCGTCTTAG CCGCTGGTAT CACAATGCTA CTAACAGACC GCAACCTAAA
M. pacificus         CCGGTCTTAG CCGCTGGGAT CACGATGTTA TTAACAGACC GAAACCTAAA
I. isolepis          CCCGTCTTAG CCGCTGGCAT TACAATGCTA CTTACAGACC GCAACCTAAA
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P. dentatus          TACAACCTTC TTTGACCCTG CAGGAGGGGG CGACCCAATT CTCTACCAAC
P. americanus        CACAACCTTC TTTGACCCTG CCGGAGGGGG TGACCCCATC CTCTACCAAC
E. zachirus          CACAACCTTC TTTGACCCTG CCGGAGGAGG AGACCCCATC CTCTACCAAC
H. elassodon         CACAACTTTC TTTGACCCTG CCGGAGGGGG TGATCCCATC CTCTATCAAC
R. hippoglossoides   CACAACCTTT TTTGACCCCG CCGGAGGTGG TGACCCCATC CTCTATCAAC
L. ferruginea        CACAACCTTC TTTGACCCTG CTGGAGGAGG TGACCCCATC CTCTACCAAC
M. pacificus         CACAACGTTT TTTGACCCTG CCGGAGGAGG TGACCCCATC CTTTATCAAC
I. isolepis          CACAACCTTC TTTGACCCTG CCGGAGGAGG AGACCCCATC CTCTACCAAC

P. dentatus          ACCTATTCTG ATTCTTTGGG CACCCCGAGG TATACATTTT GATTCTCCCA
P. americanus        ACCTATTCTG ATTCTTTGGG CACCCAGAAG TTTACATTTT AATTCTTCCA
E. zachirus          ACCTATTCTG ATTCTTTGGG CACCCAGAAG TATACATTTT AATTCTTCCA
H. elassodon         ACCTATTCTG ATTCTTTGGT CACCCAGAGG TATACATTTT AATTCTTCCT
R. hippoglossoides   ACCTATTCTG ATTCTTTGGC CACCCAGAGG TATATATTCT CATTCTTCCA
L. ferruginea        ACCTATTCTG ATTCTTTGGT CACCCAGAAG TATACATTTT AATTTTACCA
M. pacificus         ACCTATTCTG ATTCTTTGGC CACCCAGAAG TATACATTCT CATTCTCCCA
I. isolepis          ACCTATTCTG ATTTTTTGGG CATCCAGAAG TATACATTTT AATTCTCCCA


P. dentatus          GGCTTCGGTA TAATCTCACA CATCGTCGCA TACTACGCAG GGAAAAAAGA
P. americanus        GGCTTCGGGA TGATTTCTCA CATCGTTGCA TATTACGCAG GTAAGAAAGA
E. zachirus          GGCTTCGGAA TGATTTCCCA CATTGTTGCA TATTATGCAG GTAAAAAAGA
H. elassodon         GGCTTCGGGA TGATTTCTCA CATTGTTGCA TACTATGCAG GTAAGAAAGA
R. hippoglossoides   GGCTTCGGAA TAATTTCCCA CATTGTTGCA TACTATGCAG GTAAAAAGGA
L. ferruginea        GGCTTCGGAA TGATTTCCCA TATTGTTGCA TACTATGCAG GTAAAAAAGA
M. pacificus         GGCTTTGGAA TGATTTCCCA CATTGTTGCA TACTATGCAG GTAAAAAAGA
I. isolepis          GGTTTCGGAA TGATTTCCCA CATTGTTGCA TACTATGCAG GTAAAAAAGA

P. dentatus          ACCCTTTGGC TATATAGGAA TAGTTTGAGC TATAATAGCT ATTGGCCTTC
P. americanus        ACCCTTTGGT TACATGGGAA TAGTCTGGGC TATGATGGCT ATCGGACTCC
E. zachirus          ACCCTTTGGT TACATGGGAA TAGTCTGGGC TATGATGGCT ATTGGACTCC
H. elassodon         ACCCTTCGGC TACATAGGAA TAGTCTGGGC TATGATAGCT ATTGGACTTC
R. hippoglossoides   ACCTTTTGGT TACATAGGAA TAGTCTGAGC TATAATAGCC ATTGGACTCC
L. ferruginea        ACCCTTTGGC TACATAGGAA TAGTCTGAGC TATGATGGCT ATTGGACTCC
M. pacificus         ACCCTTTGGA TACATGGGCA TGGTCTGAGC TATGATGGCT ATTGGACTTC
I. isolepis          ACCCTTTGGT TACATAGGAA TAGTCTGGGC CATGATGGCT ATTGGACTCC

P. dentatus          TGGGCTTTAT TGTATGAGCC CATCATATGT TCACAGTCGG AATAGATGTG
P. americanus        TGGGCTTCAT TGTATGAGCC CATCACATGT TTACAGTCGG AATAGATGTG
E. zachirus          TAGGCTTCAT CGTCTGGGCC CATCACATGT TTACGGTCGG AATAGATGTA
H. elassodon         TGGGCTTCAT CGTATGGGCC CATCACATAT TTACAGTTGG TATGGATGTG
R. hippoglossoides   TGGGATTCAT TGTATGGGGC CATCACATGT TTACAGTCGG GATAGACGTC
L. ferruginea        TGGGCTTCAT CGTATGGGCC CACCACATGT TTACAGTCGG AATAGACGTG
M. pacificus         TAGGGTTCAT CGTTTGAGCT CACCACATGT TTACAGTCGG GATAGATGTG
I. isolepis          TGGGCTTCAT CGTATGGGCC CATCACATGT TTACAGTTGG AATGGATGTG

P. dentatus          GATACACGGG CTTACTTTAC ATCCGCAACA ATGATTATTG CAATCCCAAC
P. americanus        GACACACGGG CCTATTTTAC CTCTGCCACA ATAATTATTG CCATTCCAAC
E. zachirus          GACACACGAG CCTACTTTAC CTCAGCCACA ATAATTATTG CCATCCCAAC
H. elassodon         GACACACGGG CCTACTTTAC TTCTGCCACA ATAATTATTG CCATCCCAAC
R. hippoglossoides   GACACACGAG CCTACTTCAC TTCCGCCACT ATAATTATTG CAATCCCGAC
L. ferruginea        GACACACGAG CCTACTTTAC CTCGGCCACA ATAATTATTG CCATCCCAAC
M. pacificus         GACACGCGAG CCTACTTTAC CTCTGCCACA ATAATTATTG CAATCCCAAC
I. isolepis          GACACACGAG CCTACTTTAC CTCAGCCACA ATAATTATTG CCATCCCAAC
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P. dentatus         AGGCGTAAAA GTATTCAGCT GACTTGCAAC CCTTCATGGA GGAAATATCA
P. americanus       CGGCGTAAAA GTCTTTAGCT GACTCGCAAC CCTTCATGGA GGAAGCATTA
E. zachirus         CGGCGTAAAA GTCTTTAGCT GACTGCGCAC CCTTCATGGG GGAAGCATTA
H. elassodon        CGGGGTAAAA GTCTTTAGCT GACTTGCAAC CCTCCATGGA GGAAGCATCA
R. hippoglossoides  CGGCGTAAAA GTCTTTAGCT GACTCGCAAC CCTCCACGGG GGAAGCATCA
L. ferruginea       CGGCGTAAAA GTCTTTAGCT GACTGGCAAC CCTTCACGGG GGAAGCATTA
M. pacificus        CGGCGTAAAA GTCTTTAGCT GACTCGCAAC CCTACACGGG GGTAATATTA
I. isolepis         TGGCGTAAAA GTCTTTAGCT GACTCGCAAC CCTCCATGGG GGAAGCATCA

P. dentatus         AATGAGAGAC ACCACTACTA TGAGCCATCG GGTTCATTTT CCTCTTTACA
P. americanus       AATGAGAAAC CCCATTCCTG TGGGCCCTAG GGTTTATTTT CCTATTTACA
E. zachirus         AATGGGAGAC CCCCCTTCTC TGGGCGCTTG GCTTTATTTT CCTATTTACA
H. elassodon        AATGAGAAAC CCCACTTCTC TGGGCTCTAG GCTTTATTTT CCTATTTACA
R. hippoglossoides  AATGAGAAAC ACCACTTCTT TGGGCCCTCG GCTTTATTTT CCTCTTTACA
L. ferruginea       AATGAGAGAC CCCACTTCTA TGAGCCCTGG GCTTTATCTT CCTATTCACG
M. pacificus        AATGAGAGAC ACCACTTTTA TGAGCCCTCG GCTTTATTTT CCTATTTACA
I. isolepis         AATGAGAGAC CCCCCTTCTC TGGGCGCTTG GCTTTATTTT CCTATTTACA

P. dentatus         GTAGGAGGCC TAACGGGAAT TGTCCTGGCT AACTCCTCCC TAGATATTGT
P. americanus       GTGGGAGGTC TTACTGGTAT TGTCTTAGCT AACTCGTCTC TTGACATTGT
E. zachirus         GTCGGGGGTC TAACTGGTAT TGTCTTAGCT AACTCCTCTC TTGATATTGT
H. elassodon        GTGGGCGGCC TTACTGGCAT TGTCTTAGCT AACTCCTCTC TCGACATCGT
R. hippoglossoides  GTAGGCGGTC TTACTGGCAT TGTCTTGGCT AACTCCTCTC TTGACATTGT
L. ferruginea       GTTGGAGGCC TAACTGGAAT TGTCTTAGCT AATTCTTCTC TTGACATTGT
M. pacificus        GTAGGAGGCC TTACTGGTAT TGTCTTGGCT AATTCTTCTC TGGACATTGT
I. isolepis         GTCGGGGGTC TAACTGGTAT TGTCTTAGCT AACTCCTCTC TTGATATTGT

P. dentatus         TCTTCATGAC ACATACTACG TAGTTGCCCA CTTCCACTAC GTTCTTTCGA
P. americanus       GCTTCATGAC ACATACTATG TAGTAGCCCA CTTCCACTAT GTCTTATCTA
E. zachirus         TCTTCATGAC ACATACTACG TAGTAGCCCA CTTTCACTAT GTCTTATCTA
H. elassodon        TCTCCATGAC ACATACTATG TAGTAGCCCA TTTCCACTAT GTATTATCTA
R. hippoglossoides  TCTGCATGAC ACATACTATG TAGTAGCCCA CTTCCACTAT GTACTATCTA
L. ferruginea       CCTTCATGAC ACATATTATG TAGTAGCCCA CTTCCACTAT GTCTTATCTA
M. pacificus        TTTACACGAC ACATACTATG TAGTAGCCCA CTTCCACTAT GTCCTATCTA
I. isolepis         TCTTCATGAC ACATACTACG TAGTAGCCCA CTTCCACTAT GTCTTATCTA

P. dentatus         TAGGAGCTGT CTTTGCCATC GTTGCCGCTT TCGTTCACTG ATTCCCCCTA
P. americanus       TGGGGGCTGT ATTTGCGATC GTTGCCGCTT TCGTTCACTG GTTCCCACTA
E. zachirus         TGGGGGCTGT ATTTGCAATT GTTGCTGCCT TCGTACACTG GTTCCCGCTA
H. elassodon        TGGGTGCTGT GTTTGCAATC CTTGCCGCCT TCGTACACTG ATTCCCCCTA
R. hippoglossoides  TGGGTGCTGT ATTCGCAATC GTTGCCGCCT TCGTCCACTG ATTCCCACTA
L. ferruginea       TGGGGGCTGT ATTTGCAATC GTTGCCGCCT TCGTGCACTG ATTCCCGCTA
M. pacificus        TGGGTGCTGT CTTTGCAATC GTTGCCGCCT TCGTCCACTG ATTCCCTCTA
I. isolepis         TGGGGGCTGT ATTTGCAATT GTTGCTGCCT TCGTACACTG GTTCCCGCTA

P. dentatus         TTTACAGGTT ACACACTCCA CTCGACGTGA ACAAAAATCC ACTTCGGGGT
P. americanus       TTTACAGGCT ATACTCTCCA CTCCACATGA ACAAAAATTC ACTTCGGCCT
E. zachirus         TTTACAGGCT ATACCCTCCA CTCCACATGA ACGAAAATCC ACTTTGGCCT
H. elassodon        TTTACAGGCT ACACCCTTCA CTCCACGTGA ACAAAAATCC ACTTTGGCCT
R. hippoglossoides  TTTACAGGTT ATACCCTCCA CTCCACATGA ACAAAAATCC ATTTCGGCCT
L. ferruginea       TTTACAGGCT ACACCCTTCA CTCCACATGA ACAAAAGTCC ACTTTGGACT
M. pacificus        TTTACAGGCT ACACCCTGCA CTCCACATGA ACAAAAATCC ACTTCGGCCT
I. isolepis         TTTACAGGCT ACACCCTCCA CTCCACATGA ACGAAAGTCC ATTTTGGCCT
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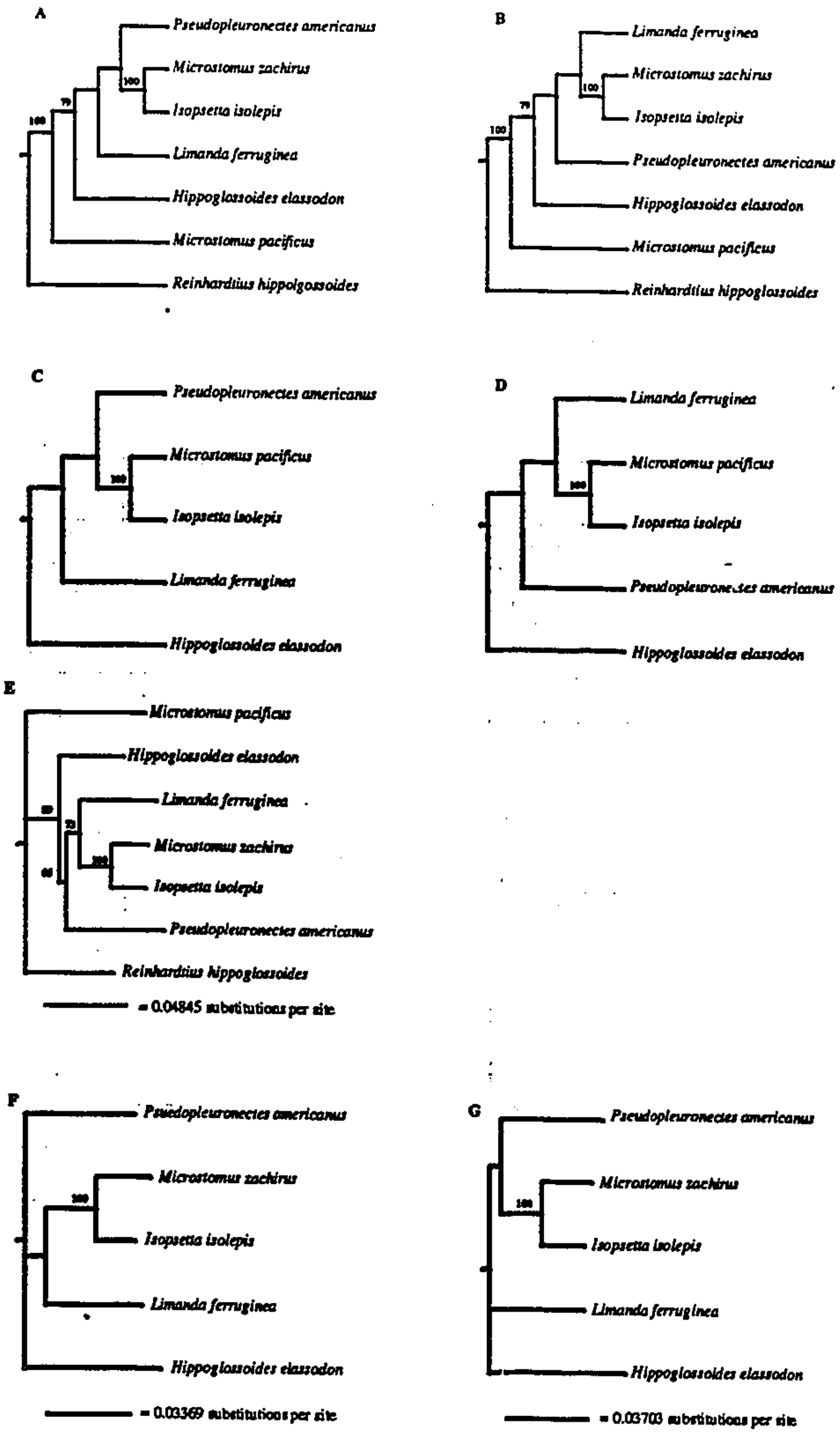
P. dentatus	TATACTTATT	GGG
P. americanus	AATGTTTGTG	GGG
E. zachirus	AATGTTCATT	GGC
H. elassodon	AATGTTTGTA	GGA
R. hippoglossoides	GATATTTATT	GGG
L. ferruginea	AATATTTGTA	GGT
M. pacificus	AATATTCATT	GGA
I. isolepis	AATGTTCGTA	GGG

Appendix IV Phylogenetic trees produced by parsimony and distance analyses of COI sequence using *Reinhardtius hippoglossoides* and *Hippoglossoides elassodon* as outgroups. Figures A and B: equally parsimonious trees obtained by exhaustive search with *R. hippoglossoides* as outgroup. Figures C and D: equally parsimonious trees obtained by exhaustive search with *H. elassodon* as outgroup. Figure E: neighbour joining tree based on Kimura two parameter distances with *R. hippoglossoides* as outgroup. Distances were calculated using all codon positions. Figures F and G: neighbour joining trees based on Kimura two parameter distances with *H. elassodon* as outgroup. Distances were calculated using all codon positions. Only bootstrap values greater than 50% are shown in all trees.


A
Pseudopleuronectes americanus
Microstomus zachirus
Isopsetta isolepis
Limanda ferruginea
Hippoglossoides elassodon
Microstomus pacificus
Reinhardtius hippolgossoides
100
79
100
B
Limanda ferruginea
Microstomus zachirus
Isopsetta isolepis
Pseudopleuronectes americanus
Hippoglossoides elassodon
Microstomus pacificus
Reinhardtius hippoglossoides
100
79
100
C
Pseudopleuronectes americanus
Microstomus pacificus
Isopsetta isolepis
Limanda ferruginea
Hippoglossoides elassodon
100
D
Limanda ferruginea
Microstomus pacificus
Isopsetta isolepis
Pseudopleuronectes americanus
Hippoglossoides elassodon
100
E
Microstomus pacificus
Hippoglossoides elassodon
Limanda ferruginea
Microstomus zachirus
Isopsetta isolepis
Pseudopleuronectes americanus
Reinhardtius hippoglossoides
= 0.04845 substitutions per site
F
Psuedopleuronectes americanus
Microstomus zachirus
Isopsetta isolepis
Limanda ferruginea
Hippoglossoides elassodon
100
= 0.03369 substitutions per site
G
Pseudopleuronectes americanus
Microstomus zachirus
Isopsetta isolepis
Limanda ferruginea
Hippoglossoides elassodon
100
= 0.03703 substitutions per site