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The Phylogenetic Position of the Genus *Atheresthes* (Pleuronectidae) and its Classification : A molecular phylogenetic approach using mitochondrial sequence data

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Abstract

The phylogenetic position of the genus *Atheresthes*, long thought to be closely related to *Reinhardtius* and included the latter by a recent study, was examined from sequence data of the mitochondrial cytochrome *b* coding region. The nucleotide sequences of 1,140 bp were determined and compared for 11 species, including two outgroup taxa. A total of 464 sites (40.7%) were variable among the species. The pairwise nucleotide differences among ingroup species ranged from 7.5% to 20.8%, those between *A. evermanni* and the other pleuronectids examined being extremely high (19.1–20.8%) and similar to the values between an outgroup species (*Paralichthys olivaceus*) and all pleuronectids examined (18.4–20.4%). Phylogenetic analyses by maximum-parsimony (MP) and maximum-likelihood (ML) methods showed that the pleuronectids did not form a single monophyletic group, at the same time supporting the monophyly of all of the latter except *A. evermanni*, with high confidence levels (94% and 91%, respectively). The unrooted MP tree resulted in a branch length to *A. evermanni* much longer than those among other ingroup species, the results overall indicating that *A. evermanni* was not closely related to *Reinhardtius hippoglossoides* and was best placed in a basal position in pleuronectid phylogeny. This supports retention of the above two genera.

Key words : Phylogenetic position, *Atheresthes*, *Reinhardtius*, Classification, Mitochondrial DNA, Cytochrome *b* sequence

The genus *Atheresthes* Jordan and Gilbert, 1880 was established to include two large (>1 m total length) flatfishes, *A. stomias* and *A. evermanni*, which are distributed around the North Pacific Ocean and Bering Sea. Norman (1934), on the basis of external morphology, suggested that the genus was related to *Reinhardtius* Gill, 1861, which included a similarly large species, *R. hippoglossoides*. Recently, however, based on a cladistic analysis of comparative morphological data, Cooper and Chapleau (1998) recognized the three species as a monophyletic group and included them all in *Reinhardtius*, supported by four synapomorphies. However, their conclusion was not reliable because their resultant phylogenetic tree comprised 128 equally parsimonious trees under the 50% majority rule. Therefore, it is necessary to confirm if the two genera are in fact sister groups.

In recent years, molecular phylogenetic analyses, using mitochondrial DNA (mtDNA) sequence data, have been adopted in fish systematics for the estimation of phylogenetic relationships (e.g., Kocher and Stepien, 1997; Miya and Nishida, 1996; 2000). The mitochon-

drial genome generally proceeds to the next generation via the maternal mitochondrion without recombination, maternal phylogenies of organisms therefore being able to be traced by examining mtDNA lineages. Thus, mtDNA should be an excellent phylogenetic marker. The present study addresses the above problem by mtDNA sequencing, the objectives being to elucidate the phylogenetic position of *Atheresthes* and its relationship to *Reinhardtius*, and to discuss the taxonomic implications.

Materials and Methods

Fish samples and DNA extraction

Mitochondrial DNA sequences were obtained from specimens of nine pleuronectid species, including *A. evermanni* and *R. hippoglossoides*, and two pleuronectiform outgroup taxa as shown in Table 1. In order to examine intraspecific variation, two specimens each of *A. evermanni*, *Lyopsetta exilis*, *Hippoglossus stenolepis* and *Psettichthys melanostictus* were examined. From all live specimens collected, a piece of skeletal

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Table 1. Specimens examined in the present study. Scientific names follow Norman (1934).

Scientific name	Specimens examined	Abbreviation
Ingroup taxa		
<i>Atheresthes evermanni</i>	HUMZ 172188, 172189	<i>Aever</i>
<i>Reinhardtius hippoglossoides</i>	HUMZ 172253	<i>Rhipp</i>
<i>Acanthopsetta nadeshnyi</i>	HUMZ 172187	<i>Anade</i>
<i>Eopsetta grigorjewi</i>	HUMZ 172192	<i>Egrig</i>
<i>Hippoglossoides dubius</i>	HUMZ 172197	<i>Hdubi</i>
<i>Hippoglossus stenolepis</i>	HUMZ 172199, 172200	<i>Hsten</i>
<i>Lyopsetta exilis</i>	HUMZ 172214, 172215	<i>Lexil</i>
<i>Psettichthys melanostictus</i>	HUMZ 172244, 172245	<i>Pmela</i>
<i>Verasper moseri</i>	HUMZ 172254	<i>Vmose</i>
Outgroup taxa		
<i>Paralichthys olivaceus</i>	HUMZ172257	<i>Poliv</i>
<i>Psetta maxima</i>	One uncataloged specimen	<i>Pmaxi</i>

muscle was removed immediately from the blind side and preserved in 99.5% ethanol. In other cases, muscle samples were taken from fresh or frozen specimens and kept in 70.0–99.5% ethanol. Voucher specimens are deposited in the Laboratory of Marine Zoology, Faculty of Fisheries, Hokkaido University, Hakodate (HUMZ). Generic names follow Norman (1934).

Total genomic DNA was isolated from 10–20 mg of muscle tissue from each sample. Tissue was digested for three hours to overnight as necessary at 37°C in 500 μ l extraction buffer (10 mM Tris, pH 8.0, 2 mM EDTA and 1% SDS) with 5 μ l proteinase K (10 mg/ml). DNA was purified through phenol-chloroform extractions and 99.5/70.0% ethanol precipitations.

DNA amplification and sequencing

DNA amplification was performed via the polymerase chain reaction (PCR) using oligodeoxynucleotide primers specific for two regions covering the whole cytochrome *b* (Cyt *b*) gene on the mtDNA genome. The primers used for the first segment (approximately 610–830 base-pairs (bp) long), including the 5' half of the gene, were as follows: L14724, 5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3' (Meyer et al., 1990); L14724B, 5'-TGA CTT GAA RAA CCA YCG TTG-3' (Palumbi, 1996); L14733, 5'-AAC CAC CGT TGT TAT TCA AC-3' (Miya, unpublished); L14734, 5'-AAC CAC CGT TGT TAT TCA ACT-3' (Inoue and Nishida, unpublished); H15341, 5'-TTT GAT CCT GTT TCA TGG AGR AA-3' (Aoyama and Nishida, unpublished); H15557, 5'-GGC AAA TAG GAA RTA TCA YTC-3' (Inoue and Nishida, unpublished). Those used for the second segment (about 680–710 bp long), including the 3' half of the Cyt *b* gene, were as follows: L15285, 5'-CCC TAA CCC GVT

TCT TYG C-3' (Inoue and Nishida, unpublished); H15966, 5'-AGG GGT GGG AGT TAA AAT CTC-3' (Palumbi, 1996); H15990, 5'-AGT TTA ATT TAG AAT CYT GGC TTT GG-3' (Aoyama and Nishida, unpublished). L and H denote light and heavy strands, respectively. Numbers given for each primer refer to the position of the 3' end of the oligonucleotide with reference to the human mtDNA sequence (Anderson et al., 1981).

PCR amplification was carried out in a total of 25 μ l volumes containing 2.5 μ l of 10 \times Ex Taq buffer with 20 mM Mg²⁺ (TaKaRa), 0.2 mM each dNTP, 0.5 μ M of each primer, 0.6 units of Taq polymerase (TaKaRa Ex Taq) and 1 μ l template. PCR conditions comprised 30–35 cycles of denaturation (94°C, 15 sec), annealing (50–55°C, 15 sec) and extension (72°C, 30 sec) on a Perkin Elmer 9700 thermal cycler. PCR products were electrophoresed on a 1.5% agarose gel, and then stained with ethidium bromide for band characterization via ultra violet transillumination.

Amplified double-stranded DNA was purified using the QIAquick PCR Purification Kit (QIAGEN) or PCR Product Pre-Sequencing Kit (USB), and sequenced directly using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer) on an automated DNA sequencer (ABI 373S or 377, Perkin Elmer). Primers used for the sequencing were the same as those for PCR. All final sequences were obtained from both strands for verification.

Phylogeny estimation

The DNA sequences were edited with the multiple sequence editor DNASIS (Hitachi Software Engineering Co. Ltd.) and aligned by eye. Phylogenetic relationships among species were estimated by the maximum-

parsimony (MP) and maximum-likelihood (ML) methods using PAUP*4.0b2 (Swofford, 1999). In the MP analysis, uninformative sites were ignored. In the ML analysis, the two substitution types model (HKY85; Hasegawa et al., 1985) was used. All parameters of the model were estimated by PUZZLE 4.0 (Strimmer and von Haeseler, 1997). To evaluate the robustness of the internal nodes of the MP and ML trees, 1,000 bootstrap (Felsenstein, 1985) and quartet puzzling (Strimmer and von Haeseler, 1997) replications were conducted, respectively. MacClade 3.08 (Maddison and Maddison, 1992) was used in various phases through the present analyses, such as preparing data matrices, exporting tree files, comparing two alternative trees site by site and exploring alternative tree topologies.

Results

Sequence variation of the *Cyt b* gene

From all 11 species, including the two outgroups, complete sequences encoding the *Cyt b* gene were obtained. As a result of sequence alignment, all of the sequences were 1,140 bp long, except for the termination codon (Appendix). Sequence differences between individuals of each species were found to be negligible, ranging from 0.0% to 0.8%.

Of the 1,140 bp aligned sequences, 676 (59.3%) sites were invariant. Of the remaining 464 variant sites, 332 (29.1%) sites were phylogenetically informative. The average nucleotide composition deviated from 25%, with A=23.1%, C=30.9%, G=15.9% and T=30.1%. Such a level of deviation is normal for fish mitochondrial *Cyt b* (Lydeard and Roe, 1997).

Uncorrected pairwise percentage differences and the numbers of transition and transversion differences

among the species examined are shown in Table 2. The percentage differences within the ingroup ranged from 7.5% to 20.8%, whereas those between the ingroup and outgroup ranged from 18.4% to 26.7%. The differences between *A. evermanni* and other ingroup taxa (19.1–20.8%) were much greater than those among other ingroup taxa (7.5–15.6%), being similar to the values between *Paralichthys olivaceus*, one of the outgroup taxa, and all pleuronectids (18.4–20.4%). In addition, the difference between *A. evermanni* and *R. hippoglossoides* was 19.6%, slightly greater than that between *P. olivaceus* and *R. hippoglossoides* (18.8%).

The numbers of transition and transversion differences at each of the first, second and third codon positions were plotted against the corrected genetic distance using the HKY85 model (Fig. 1). Substitutions at the first and second positions and transversions at the third position were almost linear to the genetic distance, whereas transitions at the third position were clearly saturated. Therefore, in the subsequent phylogenetic analysis, MP analysis was done with a data set excluding the third position transitions.

Phylogenetic relationships among taxa

MP analysis using the heuristic algorithm (100 random replications) produced a single most parsimonious tree (Fig. 2), with a tree length of 373 [consistency index (CI)=0.558; retention index (RI)=0.446; rescaled consistency index (RC)=0.249]. The topology of the ML tree was almost congruent with that of the MP tree. In the latter, all pleuronectids were not represented as monophyletic, *A. evermanni* being connected to one of the outgroup species, *P. olivaceus*. The monophyly of *A. evermanni* and *P. olivaceus* was supported by MP (83%), but not by ML (less than 50%). The monophyly

Table 2. Numbers of nucleotide differences (above diagonal) and uncorrected percentage differences (below diagonal) among examined species.

	1	2	3	4	5	6	7	8	9	10	11
1. <i>Pmaxi</i>	—	160/127	155/131	182/100	162/112	161/113	171/109	176/111	179/125	170/128	175/123
2. <i>Poliv</i>	0.2518	—	145/80	160/73	129/81	143/82	136/78	139/74	149/82	139/89	139/86
3. <i>Aever</i>	0.2509	0.1974	—	152/73	137/85	140/82	141/82	138/80	153/84	143/83	153/84
4. <i>Vmose</i>	0.2474	0.2044	0.1974	—	124/32	140/29	110/27	109/19	134/39	121/48	135/43
5. <i>Lexil</i>	0.2404	0.1842	0.1947	0.1368	—	101/45	89/37	102/39	115/51	116/56	119/53
6. <i>Egrig</i>	0.2404	0.1974	0.1947	0.1483	0.1281	—	111/36	105/30	123/46	114/53	126/48
7. <i>Rhipp</i>	0.2456	0.1877	0.1956	0.1202	0.1105	0.1290	—	73/26	113/50	107/55	112/44
8. <i>Hsten</i>	0.2518	0.1868	0.1912	0.1123	0.1237	0.1184	0.0868	—	114/40	103/49	104/46
9. <i>Hdubi</i>	0.2667	0.2026	0.2079	0.1518	0.1456	0.1483	0.1430	0.1351	—	67/19	105/36
10. <i>Anade</i>	0.2614	0.20	0.1983	0.1483	0.1509	0.1465	0.1421	0.1333	0.0754	—	114/41
11. <i>Pmela</i>	0.2614	0.1974	0.2079	0.1561	0.1509	0.1526	0.1368	0.1316	0.1237	0.1360	—

Values above diagonal indicate total numbers of transitions (left) and transversions (right) in 1,140 bp sequences.

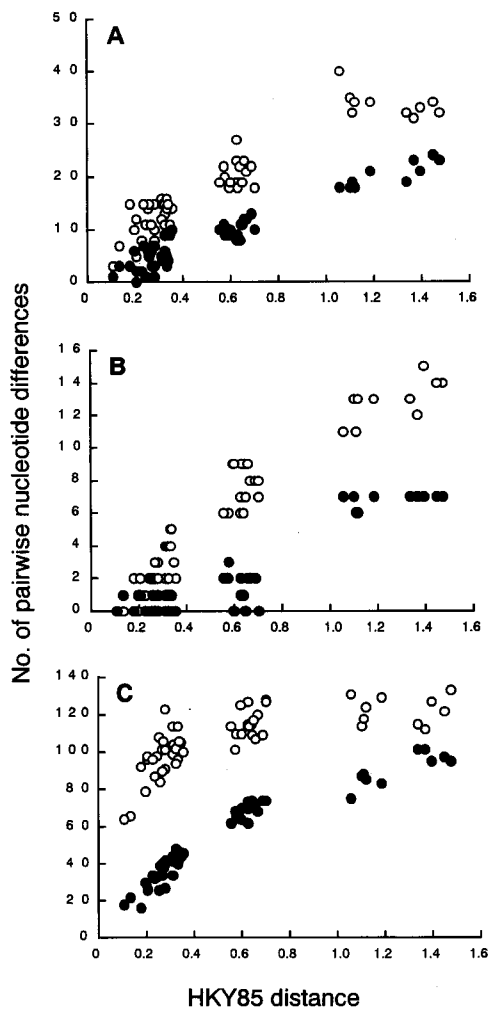


Fig. 1. Pairwise nucleotide differences at each codon position: (A), first, (B) second and (C) third positions. Numbers of transitions (○) and transversions (●) plotted against corrected genetic distance using the Hasegawa-Kishino-Yano (1985) substitution model.

of all pleuronectids, except for *A. evermanni*, was strongly supported in both the MP and ML analyses (94% and 91%, respectively), although most of the relationships within this group remain unresolved.

In the unrooted MP phylogram, branch lengths between *A. evermanni* and the remaining pleuronectids were much longer than those among species within the latter group (Fig. 3). Accordingly, a close relationship between *A. evermanni* and *R. hippoglossoides* can not be postulated.

Discussion

Phylogenetic position of *Atheresthes*

The present mtDNA analysis indicated that *A. evermanni* was phylogenetically distantly related to all of the other pleuronectid fishes, and that *A. evermanni* and

R. hippoglossoides did not constitute a monophyletic group (Figs. 2 and 3). Although additional analyses using more basal taxa as outgroups are necessary for determining the phylogenetic position of *A. evermanni* with any degree of certainty, the genus *Atheresthes* is apparently located at the most basal position within Pleuronectidae at least. Since the branch length to *A. evermanni* from major pleuronectids is as long as those to *Psetta maxima* and *P. olivaceus* (Fig. 3), *Atheresthes* may have diverged from other representatives early in the evolution of pleuronectid fishes. The genus *Reinhardtius*, on the other hand, appears to be more closely related to *Eopsetta*, *Hippoglossus* and other relatively small species, rather than *Atheresthes*.

Classification

The present study indicated strongly that *A. evermanni* and *R. hippoglossoides* are not sistergroups of each other, a result supporting previous classifications which recognized two genera for these species (e.g., Norman, 1934; Sakamoto, 1984; Nelson, 1994). *Reinhardtius sensu* Cooper and Chapleau (1998) should not be retained.

A. stomias, used as a representative of *Atheresthes* by Cooper and Chapleau (1998) but not available for this study, is possibly distinguishable from *Reinhardtius* on the basis of morphological features. Norman (1934) described two species of *Atheresthes* as having a narrower interorbital space than *Reinhardtius*, and Ahlstrom et al. (1984) later indicated that larvae of *A. stomias* had preopercular spines and a spinous supraocular crest, whereas those of *R. hippoglossoides* did not. These observations suggest that *A. stomias* is also phylogenetically distant to *R. hippoglossoides*. Further investigations, using all three species of the two genera and more ancestral outgroup taxa, should define *Atheresthes* and *Reinhardtius* more thoroughly.

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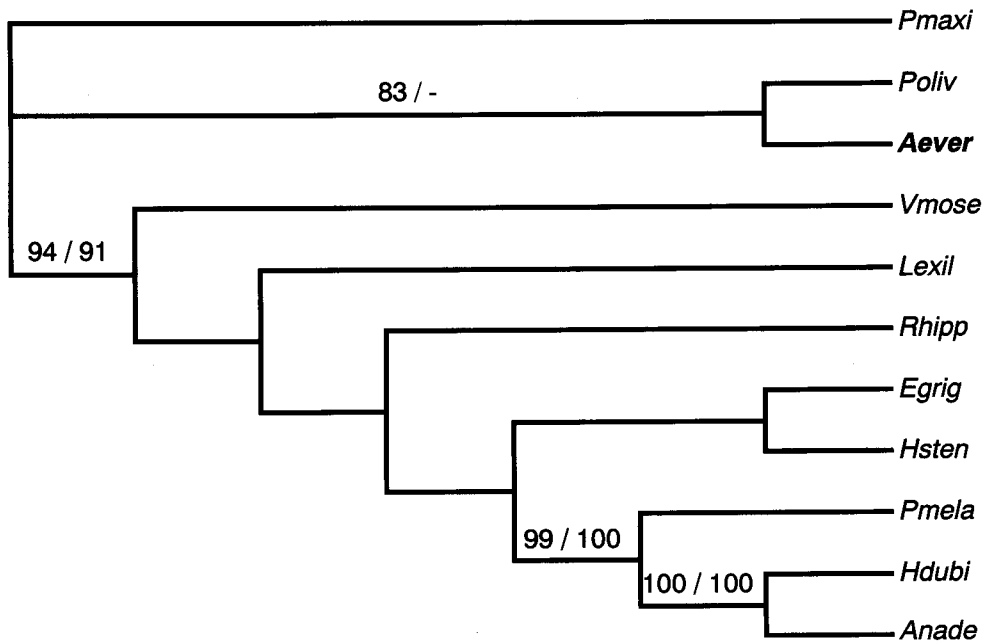


Fig. 2. Single most parsimonious tree of nine pleuronectids and two outgroups. Numbers above branches indicate bootstrap (in MP)/quartet puzzling (in ML) values greater than 50% obtained for 1,000 replicates each. Taxa abbreviated as in Table 1.

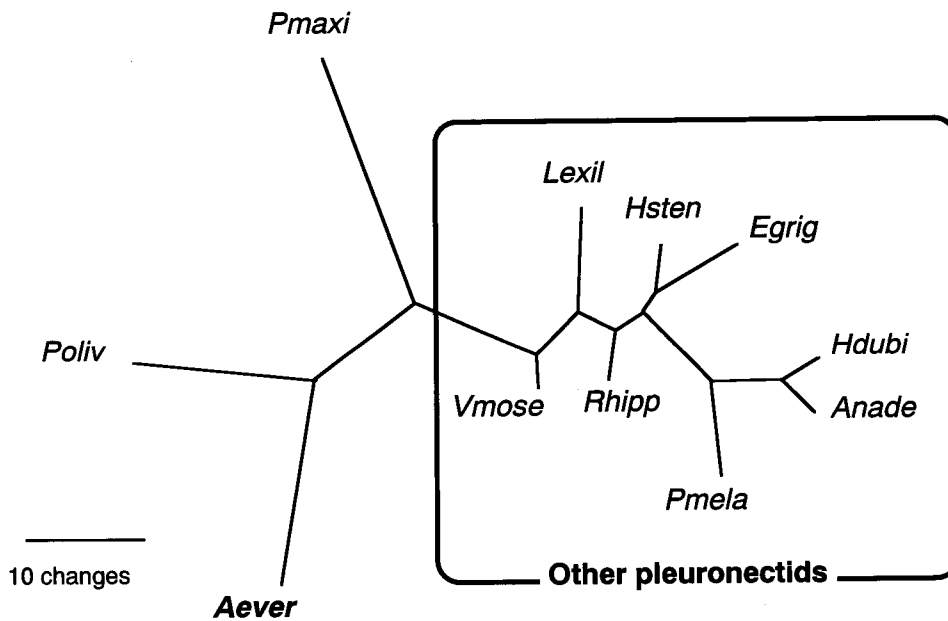


Fig. 3. The unrooted most parsimonious phylogram of all examined species. Branch lengths are proportionate to numbers of substitutions. Taxa abbreviated as in Table 1.

References

- Ahlstrom, E.H., Amaoka, K., Hensley, D.A., Moser, H.G. and Sumida, B.Y. (1984). Pleuronectiformes: Development. p. 640-670, Moser, H.G., Richard, W.J., Cohen, D.M., Fahay, M.P., Kendall Jr., A.W. and Richardson, S. L. (eds.). *Ontogeny and systematics of fishes*. Am. Soc. Ichthyol. Herpetol. Spec. Publ. 1.
- Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A. J.H., Staden, R. and Young, I.G. (1981). Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457-465.
- Cooper, J.A. and Chapleau, F. (1998). Monophyly and intrarelationships of the family Pleuronectidae (Pleuronectiformes), with a revised classification. *Fish. Bull.*, **96**, 686-726.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783-

- 791.
- Hasegawa, M., Kishino, H. and Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.*, **21**, 160-174.
- Kocher, T.D. and Stepien, C.A. (eds). (1997). *Molecular Systematics of Fishes*. Academic Press, New York.
- Lydeard, C. and Roe, K.J. (1997). The phylogenetic utility of the mitochondrial cytochrome *b* gene for inferring relationships among Actinopterygian fishes. p. 285-303, Kocher, T.D. and Stepien, C.A. (eds). *Molecular Systematics of Fishes*. Academic Press, New York.
- Maddison, W.P. and Maddison, D.R. (1992). MacClade, Ver. 3.08. Sinauer Associates, Sunderland, Massachusetts.
- Meyer, A. and Wilson, A.C. (1990). Origin of tetrapods inferred from their mitochondrial DNA affiliation to lungfish. *J. Mol. Evol.*, **31**, 359-364.
- Miya, M. and Nishida, M. (1996). Molecular phylogenetic perspective on the evolution of the deep-sea fish genus *Cylothone* (Stomiiformes: Gonostomatidae). *Ichthyol. Res.*, **43**, 375-398.
- Miya, M. and Nishida, M. (2000). Molecular systematics of the deep-sea fish genus *Gonostoma* (Stomiiformes: Gonostomatidae): Two paraphyletic clades and resurrection of *Sigmops*. *Copeia*, **2000**, 378-389.
- Nelson, J.S. (1994). *Fishes of the World*, 3rd ed. John Wiley & Sons, New York.
- Norman, J.R. (1934). *A systematic monograph of the flatfishes (Heterosomata)*. 1. Psettodidae, Bthidae, Pleuronectidae. Brit. Mus. Nat. Hist., London.
- Palumbi, S.R. (1996). Nucleic acids II: The polymerase chain reaction. p. 205-247, Hillis, D.M., Moritz, C. and Mable, B.K. (eds). *Molecular Systematics (2nd ed.)*. Sinauer Associates, Sunderland, Massachusetts.
- Sakamoto, K. (1984). Interrelationships of the family Pleuronectidae (Pisces: Pleuronectiformes) *Mem. Fac. Fish. Hokkaido Univ.*, **31**, 95-215.
- Strimmer, K. and von Haeseler, A. (1997). PUZZLE ver. 4.0: maximum likelihood analysis for nucleotide, aminoacid, and two-state data. Zool. Inst., Univ. Muenchen, Muenchen.
- Swofford, D.L. (1999). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Ver. 4. Sinauer Associates, Sunderland, Massachusetts.

Appendix. Aligned nucleotide sequences of 1,140 bp from the mitochondrial cytochrome h gene. Taxa abbreviated as in Table 1.

		100	
Pmaxi	ATGACCAGCCTACGAAAATCCCACCCCTATCCATAAGATCGCAAATGATGCTTTAGTAGATCTCCCTGCCCCCTCTAATATCTCAGTTTGATGAAACCTTTGGCTCTCTCTGGCCCTCTGCTTAGCCACTCAACTCATTACCGGACTCTTT		[150]
Poliv	...G.....A.....CC.TT.....A.....A.....C.....T.A.A.....G.....T.....C.....G.....C.....CT.A.A.A.....T.....AT.....C.....GA.TT.A.....C.A.A.C		[150]
Aever	...A...T...T...G...A...T...CC.AT.....C.....C.....T...C.....C.....T...C...A.....G.....T...C...G...A...A...A...TC.GATT...C...A...C...G...T...CT.A...		[150]
Rhipp	...G...T...T...T.....C.T...A.....C.....T...C...A.....C.....G.....G.....A...A.....T...TA...C...A...TGC.....CT.G...		[150]
Vmose	...G...T...T...T.....CC.T...A.....C.....A.T...C.....T.....T.....G.....G.....A...T...C...G...T...A...TG...G.....CT.A...		[150]
Lexil	...G.....G.....CC.T...A...T...C.....C.....T.....A.....C.....T.....A.....A.....A.....TA.....A...TGCA.....CT.A...		[150]
Egrig	...G.....T...T.....AC...T...A.....T...C.....T...C.....C...T...C.....T...G...G.....G...C.....G...A...T...AT...C...GA.TG...T...CT.A...		[150]
Hsten	...G...T...T...T.....C.T...A.....C.....C.....C...A.....A.....T.....T.....G.....G.....T...A...A...T...ATT...C...A...TGCA.....CT.A...		[150]
Hdubi	...G...A...C...T.....CC.T...T...A.....C.....C...C...T...A.....C...C...T...C...G.....C...G.....CT.A...A...T...TG...C...GA...GC.....CT.A...C		[150]
Anade	...G...AT...C...T.....CC.T...T...A.....C.....G...G...C...A.....C...C...T...C...G.....CT.G...A...T...TT...C...GA...GC.....CT.A...C		[150]
Pmela	...G...A...C...T.....AC...T...A.....C.....C...A.....A...C...T...T...C.....G.....A...A.....TA...C...GA...GC.....CT.A...C		[150]
		200	
Pmaxi	TTAGCTATGCACATACCGCGGATATCGCAACAGCCTTTACCTCCGTCGCCCATATACCCGAGACGTTAATACCGTTGATTAATCCGAAACCTACACGCCAACGGCGCATCCTTCTTCTTCTTATGTATTATGCACATATCGGCCGA		[300]
Poliv	...C...A...C...T.A.C.....T...C...T...G...C...TG...G...A...C.....A...C...T.....A.....TA.C.C.....CCTC...C.....		[300]
Aever	C.T...C...A.....AT.C.....T...C...A.....C...TG...G...C.....A.....TA.C.....T...T...T...G.C.....CTT...C.....T...		[300]
Rhipp	C...C...A.....GT.A.....T...T...C.....G...T...C...TG...G...C...C...C...T...G.A.T...T...A...T...C...C...CCT...C.....		[300]
Vmose	...C...A.....GT.A.C.T.T.T...C...TA.T.A.....TG...G...C.....GC.T...G.A.T...T...A...T...A.T...CCTT...T...T...		[300]
Lexil	C.T...A...C...G...A.....C...T...T...C...T...TG...G...C...C...C...T...G.A.T...T...A...T...A.C...C...CCTT...C.....		[300]
Egrig	C...A...C...AT...T...T...C...T...G...G...C...C...C...C...T...GTA.T...T...A...T...A.C...C...CCTT...G.....		[300]
Hsten	C...C...A...C...AT.A.C.T.T.T...C...A...C...TGT...C...C...C...GC.T...G.A.T...T...A...T...A.T...CC.C...CCTT...T...T...		[300]
Hdubi	...C...A...C...AT.A...T...T...T...T...T...A...C...TTG...T...C...C...C...T...G.A.T...T...T...A...T...A.T...C...CCTC...T...		[300]
Anade	...C...A...AT.A...T...T...T...C...T...G...C...TGT...C...A...C...T...GTA.T...T...T...A...T...A.T...C...CCTT...T...		[300]
Pmela	...C...A...C...AT.T.C...T...T...A...C...TTG...G...C...C...C...C...T...G.A.T...T...A...T...A.T...C...CCTC...T...G		[300]
		400	
Pmaxi	GGTCTGTACTACGGCTCTTACCTTTATAAAGAAACCTGAAACGTTGGGAGTTATCCTTCTTCTCTCTGTTATAGCAACTGCCTTCGTCGGCTATGTTCTTCCCTGAGGACAAATATCTTTCTGGGGCGCAACCGTAATTACCAATCTTATG		[450]
Poliv	...A.....TT.G.....A.....T...T...C...C...G...G...A...GATG...C...T...T...T...C...C.....A.....T...C...T...C...C...C...AC.C		[450]
Aever	...C...T...T...A.....A.....C.A.C...G...A...C...C...T...A...AT...A.....A...A...A...C.....G...C...A...T...T...T...C...AC.C		[450]
Rhipp	...G...C...T...A...C.....A...CTA.T...CG...C...A...C...A...AT...G...A...C...C...C...T...G...G...T...A...T...T...C...CC.A		[450]
Vmose	...A...T...T...C...C.....A...CTA.C...G...G...A...ATG...A...T...T...A...C...C...T...G...G...T...A...T...T...C...		[450]
Lexil	...C...T...A...C.....A...CTA.C...C...A...A...A...ATG...A...A...C...C...T...G...A...A...A...T...T...C...C.A		[450]
Egrig	...C...T...A...A...T...T...G...A...C...C...A...AT...A...A...C...C...C...T...G...A...A...A...T...C...C...C.A		[450]
Hsten	...A...T...C.....C...A...CT.T.G...T...C...C...A...AT...A...T...A...C...C...C...T...G...G...T...A...T...T...C...C.A		[450]
Hdubi	...A...T...C.....G...A...CTA.C.T...G...A...A...A...ATG...C...T...T...A...C...C...C...T...G...G...T...A...T...C...C...C.A		[450]
Anade	...C...T...C...C.....G...A...CTA.T...T...CG...A...C...T...A...ATG...C...T...T...A...C...C...C...T...G...G...T...A...T...C...C...T...		[450]
Pmela	...C...C...T...C.....G...G...CAA.C...G...CG.T...G...T...A...ATG...C...T...T...G...C...C...T...G...C...A...T...C...C...C.A		[450]
		500	
Pmaxi	TCCGCGTCCCTACATTGGCACCACCCTTGTCAATGAATCTGAGGGGGCTTCTCAGTAGATAACGCAACCCCTCACCCGGTCTCTCGCCTTCCATTTTCTCCTCCGTTTGTATTGACAGTACGGTTATTACCTCCTCTCTCCTC		[600]
Poliv	...A...A...T...TG.C...T.A...A...G.....A.....G.....T...T...T...C...A.....A.....T...A...C...C...AT.C...A...C...C...CA.C...A...C...TA.T...A		[600]
Aever	...T.....TG.A...AGG...T.....C...G...T.....T...T...T...G...T...T...A...T...C...C...AT.C...C...A...C...G...C...CA...A...A...T...T...G		[600]
Rhipp	...T.....TG...A...A...C...G...T...T...T...T...C...T...T...T...T...T...T...T...C...C...TT.C...C...CA.C...C...C...CA...A...A...C...T...A...A...T...T		[600]
Vmose	...A...T...TG.C...T.A...C...C...G...T...T...T...C...T...T...T...T...T...T...T...C...C...TT.C...C...A...C...C...C...CA...A...G...C...T...T...T		[600]
Lexil	...T.....TG.C...A...C...C...T...A...T...T...C...T...T...T...T...T...T...T...A...T...C...C...TT.C...C...CC.C...C...C...CA...A...A...C...A...T...T...		[600]
Egrig	...T.....G.C...G...A...T...C...C...G...C...T...T...C...T...T...T...T...T...T...T...A...T...C...C...TT.C...C...CA.C...C...CC...AA...G...T...T...		[600]
Hsten	...T.....G.C...A...A...G...C...G...T...T...T...C...T...T...T...T...T...T...T...T...C...C...TT.C...C...CA.C...C...T...C...CA...T...A...T...T...T		[600]
Hdubi	...T...A...TG...GGG...T...C...C...T...G...C...T...T...T...C...T...A...T...A...C...C...TT.C...C...CA.C...C...C...CA...A...A...T...A...T...T...		[600]
Anade	...T...A...TG...AGG...T...C...C...T...C...T...C...C...A...C...C...T...A...C...C...TT.C...A...CA.C...C...C...CC...A...A...A...T...G		[600]
Pmela	...T...T...TG.C...GGG...A...C...C...G...T...T...T...C...T...T...T...T...T...T...T...T...A...C...C...GT.C...C...CA...A...C...A...A...A...T...T		[600]

Appendix. Continued.

| 700

Pmaxi CACGAAACCGGCTCCAATAACCCCGTAGGCCTTAACCTCCGACCGTGATAAAATTTCTTTCCACCCTTATTTTTTCATACAAAGACCTTCTCGGATTCGTCCTCTTGACAGCACTCGCAACCCTAGCTTTATTTTCCACCTAACCTACTT [750]
 PolivT.....C.....ACT..AT.G..T..A...T.A..C...G.CC.C.....A..C.C.....T.....C..G...T.CA.C...C.C.TT..C..A..TG...C.....C..C.G...TT.G [750]
 Aever ..T.....A.....C.....AC..AT.A...A...T...C..GG.C...T.....C.....C..C.....C.....G...CA..A..TC.T.TT..C..GA..T.T..C..CC.....C.....T..G..C [750]
 RhippT.....A.....T...ACC...T.A...A...T.A..C..GG..C.C.....C..CA.T.....CT.A..T..T.CA..A..C.C..T..T.A...T...C.....G...T...T..A [750]
 VmoseT.....A..C.....ACC..GT.A..T..A..TT...C..GG.CC.....C..CA.T.....G.....CT.G..C..T.CAA...TC.TG.T...AA..T...T...CC.G...C..A...TT.G [750]
 LexilA..C.....TACC..G.....A...T.C.....G.CC.C.....A..C..CA.G.....CT.A..C..T.CA..T..TC.CG.T...AA..G...C..C.....C..T..C.....CT.A [750]
 EgrigG..A..C.....ACC..G..A..T..A...T.C..C...G..C.....C..C..CA.C.....CT.A..C...CA...C.T..G...A...T...C..CC.C...T..G..T..TT.A [750]
 HstenT.....A..C.....AACC..G..A...A...T.C..C...G..C.C..T.....G...C..C..CA.T...G.....CT.A..C..T.CA...TC.T..T...T...CC.....C..A..T..CT.A [750]
 Hdubi ..T...T..G..A.....TACC..A..A...AA..T.C..C...G..CC.A..T.....C..CA.G.....T...G..C..T.CA..T..TC.T..T..C..A..CT...C..CC.C..C..C..A..T..TT.A [750]
 AnadeT..T..A..C.....ACC..A..A..T..AA..T.C.....G.CC.A.....C..C..CA.....T..A..C..T.CA..A..TC.T..T..T..G..TT.G..T..CC.....C..C..A...TT.A [750]
 PmelaG..T.....A..C.....ACG...G...A...T...C..G.CC.A.....C..CA.....G.....AT.A..T...CA..G..TC.T..T...T..A..CT...C...C..A...T..A [750]

| 800

Pmaxi GGCGACCCAGATAAATTCACACCAGCGAACCCTAGTCACTCCCCCTCACATTAAACCAGAAATGGTACTTTCTGTTTGCCTACGCCATCTTCGGTCCATCCCAATAAGCTTGGAGGGGTGCTCGCGCTACTTTTCTCCATCCTTATC [900]
 PolivT.....C..C..A...A..T..T..A..G..C..T..C..G..C..G..A..T..C..C.....A.....TC.C..A..T..T.....A...C..A..A...C..GT.G...T..TT.A... [900]
 Aever ..A.....G.....T..T..C..C..A..T..A..T..T.....A.....G..C..G...T..C..C.....A.....C...A..T...C..C..A...T...T..A...C...T.G...T..G...T..AG.. [900]
 Rhipp ..A.....A.....T..C..C..A..T..A..T..T..A.....A.....G..A...C...C...T.....T..TC.C.....C..A...G...T..T..C..TT.A..T..G...T..AG.T [900]
 Vmose ..G.....C.....T..C..T..A..T..A..T..T.....A...T..C..G.....A.....C..T...T...T...C..C.....C.....A..T..C..TT.A..T..G...TT..AG.. [900]
 Lexil ..A.....T..C..T..T..C..C..A...A..T..T..A..G..A...C.....T..C..T.....T..TC.C..C.....C..A...G...A..T..C..T..A...T..C..TT..AG.T [900]
 Egrig ..A.....A.....C...T..C..C..A...A..T...A..T..A...C..G.....C..C.....TC.C..C.....C..A...T...A..T..C..TT.A...A...T..GG.T [900]
 Hsten ..A.....T.....T..C..T..A..T...T..T.....A.....G...G..A...C.....T..TC.C..C.....T...C..A...A..A..T..C..TT.A...A...T..AG.T [900]
 Hdubi ..A.....C..C.....T..T..T..A...A..T..T..A...A..T..C..G..C...A...C..A.....C..C..C.....C..A...A...T..C..T..A...A...TT..AG.T [900]
 Anade ..A..T..T..C...T..T..T..A...A..T..T..G...A..T.....G..A...C.....TC.C..C.....C..A...A...T..C..TT..G...GG.A [900]
 Pmela ..A..T.....C..T..A...C..T...G..T..A...C..G...G..A...C..C..C.....C..C..C.....C..A..C..C...A..T..T..TT.A...CG.T [900]

| 1000

Pmaxi CTGATACTTGTAACCCTACTCCACACCTCTAAACAACGTGGACTAACCTTCGCCCCCTCTCCCAATGTTTATTCTGACTACTAGTTGCAGACGTCCTTGTCTTGACTGAATCGGGGGCATACTGTGAACATCCTTTTCATTATTATT [1050]
 Poliv ..C..G...C...A..C.....A..A...AA.T...TA...A...T..T..G...TCC...T...TCC..C..A.....AA.AA.TC.C..A.....A..G..C..A...C...TG... [1050]
 Aever ..TT.....T.....T.....G.....G.....AA.C..T.TA...A..T..AA.A...TCC.G..T...TCTT...A.....TA.AA..C.T..C...A...C..A...C..C..TG.G...C [1050]
 Rhipp ..C..G...C...T.C..T...CA.C...TG..T...T..AA.A...T..G...GTCT..G..A...TA.AA.TC.A...T...A..G..C..A...C..C...G...C... [1050]
 Vmose ..A..G..C..C...T...T...G..AA.C...TA...T..AA.A...TC...GTC.T...A...TA.AA.TC.A...T..A...A...C...C...G..C..C... [1050]
 Lexil ..C..G...C...T..TT.A...AA.TT...TA...AT.AA.A..G.T.C.C...TCT..G..A...TT.AA.TC.A...T...T..G..C..A...C..C...G...C..C [1050]
 Egrig T.A..G..C..C...C..A...AA.T..T.TA...T...A..A..G.TC..G...GTCTT...A...A.GA.TC.A..G...T..A..G..G..C..A...C..C...G...C..C [1050]
 Hsten ..T..G..C..C...T.C..T..T...AA.C..G.TA...T...AA.A...T..G...TCTT...A...TA.GA.TC.A...T..A..A..G..C..A...C...G...C... [1050]
 Hdubi ..T..G...C...T.C...T..C...G..AA.C..C.TG...TG.AA.A..G.T.C.G...GTCTT...A..T...AA.AA.TC...T..A..G...C..A..G..C...G...C... [1050]
 Anade ..T...C...C...T.C..A...T..C...GA.T..C.TA...TG.AA.A..G.TCC.G...GTCTT...A..T...AA.AA.TC...T..A..G...C..A...C...G... [1050]
 Pmela ..C..G..C..C...TT.C..A..T...C..G...AA.CT.G.TG...TG.AA.A...TCC...GTCC...A..T...GA.AA.TC.A..C...T..A..A..G..C..A...C...G...C...C [1050]

| 1100

Pmaxi GGTCAGATCGCATCAGCTCTCTACTTACCATTFTTTCTAATTTTATTCOCATTTGGCAGGCTTGTAGAAAACAAGCCCTTGCCTGATCT [1140]
 Poliv ..A..AG.T..T...TTC.....TT.C...CT..G.CA..A..T..G.AA...GAC.....T..GATT..C.GA...CAG [1140]
 Aever ..A..AG.T..C...TT.A...T..C.C.C...C.AGCTA.A.G.A..G..GAC...G..T...A...GA..GCGA [1140]
 Rhipp ..A..AG.A...CTC..T...G..C.C.C...G.CC.GA...ACA...T..AC...G..T...T...GA...AAA [1140]
 Vmose ..A..AG.A...CTC..T...T...G.TC.C..C..GG.CC.GA...ACA...T.GAC...G...T...C.GA...AAA [1140]
 Lexil ..A..AG.A...CTC..T...T..C.C.C...C.CC.TA...ACA...GAC..T...T...GA...AAA [1140]
 Egrig ..A..AG.A..G..CCTC..T..T...T..C.C.C...G.C.CA...ACA...T.GAC.G...T..G...C.GA...AAA [1140]
 Hsten ..A..AG.A..G..TCTC..T...T..C.C.C...G.CC.GA...ACA...T.GAC...G..T...T...GA...AAA [1140]
 Hdubi ..C..AG.A...CCT.A...TT..C.C.C...G.CC.TA...ACA...GAA.G...T..C.GA...AAC [1140]
 Anade ..C..AG.A...CCT.A.T...T..C.C.C...G.CC.CA...ACA...GAA.G...T...C.GA...AAC [1140]