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Utility of tRNA Genes from the Complete Mitochondrial Genome of *Psetta maxima* for Implying a Possible Sister-group Relationship to the Pleuronectiformes

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Wei Shi, Xiao-Yu Kong, Zhong-Ming Wang, and Jin-Xia Jiang (2011) Utility of tRNA genes from the complete mitochondrial genome of *Psetta maxima* for implying a possible sister-group relationship to the Pleuronectiformes. *Zoological Studies* **50**(5): 665-681. The complete sequence of the 17,583-nucleotide mitochondrial genome of *Psetta maxima* (Linnaeus, 1758) was determined, and it contains 13 protein-coding genes, 2 ribosomal (r)RNA genes (*srRNA*, *lrRNA*), 22 transfer (t)RNA genes, and a putative control region as do most other fish mitogenomes. A striking finding is that this genome contains a surprisingly long *ND2* gene of 1100 bp, which turns out to be the longest one among 700 teleost mitogenomes sequenced so far. In order to better understand the phylogenetic relationship of the Pleuronectiformes, extensive and intensive phylogenetic analyses were conducted using maximum-likelihood and Bayesian inference based on data of the 1st and 2nd codon positions of 12 concatenated protein-coding genes plus 22 stem or complete tRNA genes from 41 teleostean mitochondrial genomes. The results showed that the Pleuronectiformes is a monophyletic group, and it shared a recent common ancestor with carangids, which provides evidence for lower-percoid origins. These conclusions disagree with the hypothesis that flatfishes were derived from an ancestor species closely related to the Clupeiformes, Zeiformes, or Beryciformes. Comparisons of phylogenetic analyses between tRNA genes and protein-coding genes indicated that tRNA genes, especially unpaired regions, can provide useful and specific information for inferring phylogenetic relationships. Therefore, we suggest that tRNA genes be included when mitochondrial genomic data are used for phylogenetic analyses. The results of this study should be able to increase our understanding of the possible sister-group of the Pleuronectiformes to some extent. http://zoolstud.sinica.edu.tw/Journals/50.5/665.pdf

Key words: Phylogenetic relationship, Carangidae, tRNA gene, Mitogenome.

The Pleuronectiformes (flatfish) are remarkable fish because they undergo a dramatic metamorphosis from bilaterally symmetrical larvae to laterally compressed adults, with both eyes on one side of the head. These species are primarily distributed in marine environments, and a few inhabit freshwater and estuaries; some of these species are of great commercial importance. The order is comprised of approximately 678 extant species belonging to 134 genera in 14 families (Nelson 2006). The Pleuronectiformes has sustained many taxonomic changes over a long period of time. Initially, it was recorded by Linné (1758) as the genus *Pleuronectes*. As more species were found, the taxon was elevated to family level by Cuvier (1817). Subsequently, Günther (1862) raised it to a suborder of the Pleuronectoidei, and eventually Cope (1871) defined flatfishes as the order Heterosomata. After the order was redefined to a suborder (Jordan

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and Evermann 1898), the current taxonomy of the Heterosomata (Pleuronectiformes) was recovered by Jordan (1923). However, what the sister-group is to flatfishes is still in question. There have been many hypotheses about that. Boulenger (1901) proposed that flatfishes evolved from dories (Zeiformes), and Regan (1910) believed that they might have been derived from the Percoidei based on similarities of the fin rays and characteristics of the supramaxillary. Thereafter, Gregory (1933) postulated a likely evolutional succession as follows: (Isospondyli, (Clupeiformes (Beryciformes, (Zeiformes, Pleuronectiformes)))). Kyle (1921) and Chabanaud (1949) considered all major flatfish taxa to be independent offshoots of an evolving "preperciform" lineage. Amaoka (1969) agreed with this interpretation based on similar features of the epicentral, epimeral, hypomeral, and myorhabdoi in Bothidae, *Sardinops coerules* (Clupeiformes), and *Anaga anago* (Anguilliformes) fishes. However, Norman (1934) pointed out that flatfishes might have evolved from some typical percoids based on plesiomorphic character states, such as *Epinephelus morio* (Perciformes; Serranidae). In disagreement with Amaoka's contention, Li and Wang (1995) inferred that flatfishes might have originated from the early Clupeoidei during the Cretaceous period. This was supported by the existence of the nephrohaemal process and the absence of the nephrohaemal arch on the ventral vertebrae in some Pleuronectidae, which are similar characteristics found in the following fishes: *Anchovilla chinensis* (Clupeiformes), *Saurida filamentosa* (Aulopiformes), *Astroconger myriaster*, and *Muraenesox cinereus* (Anguilliformes). Chapleau (1993) considered lower-percoid origins for flatfishes, although specific groups representing lower-percoids were not explicitly defined until very recently (Little et al. 2010).

On the other hand, although molecular phylogenetic analyses of the sister-group to the Pleuronectiformes were explored in some studies, most of those focused on phylogenetic relationships within the Acanthomorpha or Teleostei, and only a few flatfish species were from the Pleuronectiformes (Miya et al. 2003 2005, Dettai and Lecointre 2005). Other molecular phylogenetic studies were devoted to intra- and inter-family relationships within the order using DNA fragment analyses (Tinti et al. 1999, Berendzen and Dimmick 2002, Infante et al. 2004, Pardo et al. 2005, Kartavtsev et al. 2008, Sharina and Kartavtsev 2010). However, no particular report on exploring the sister-group to the flatfishes using molecular markers has emerged to date.

The complete mitochondrial genome of *Psetta maxima* was determined in this study, and was subsequently used for phylogenetic analyses together with other mitogenomic sequences from 10 flatfishes and 31 other species that fully represent the 6 orders hypothesized to be related as a sister-group to flatfishes. Datasets for the phylogenetic analyses included the concatenated protein-coding sequences (CDSs, excluding the *ND6* gene) and transfer (t)RNA sequences.

In recent studies, when complete mitochondrial (mt)DNA sequences were employed for phylogenetic analyses, tRNA gene sequences were generally ignored or only the stem regions were used, due to their different modes and rates of evolution (Miya et al. 2003 2005, Peng et al. 2006). Although Kumazawa and Nishida (1993) showed that the stem region of the mitogenomic tRNA gene was useful in resolving deep branches in animal phylogenies, it is not clear if the unpaired regions of the tRNA gene also have usefulness in phylogenetic analyses. Therefore, comparisons of phylogenetic performances between protein-coding and tRNA genes were carried out in this study to examine the utility of the stem and unpaired regions of mitogenomic tRNA genes for phylogenetic analyses.

MATERIALS AND METHODS

Sampling, DNA extraction, polymerase chain reaction (PCR), and sequencing

Psetta maxima samples were obtained from a fish farm in Haiyang County, Shandong Province, China. A portion of the epaxial musculature was excised from fresh specimens and immediately stored at -70°C. The total genomic DNA was extracted using an SQ Tissue DNA Kit (OMEGA, Guangzhou, China) following the manufacturer's protocol. Based on alignments and comparisons of complete mitochondrial sequences of flatfishes, 12 primer pairs were designed for amplification of the *P. maxima* mtDNA genome (Table 1). More than 30 bp of an overlapping fragment between the two tandem regions was used to ensure the correct assembly and integrity of the complete sequence.

The PCR was performed in a 25-μl reaction volume containing 2.0 mM $MgCl_2$, 0.4 mM of each dNTP, 0.5 μM of each primer, 1.0 U of *Taq* polymerase (Takara, Dalian, China), 2.5 μl of 10x

Taq buffer, and approximately 50 ng of a DNA template. PCR cycling conditions included initial denaturation at 95°C for 3 min, 30-35 cycles at 94°C for 45 s, an annealing temperature of 45-55°C for 45 s, and elongation at 68-72°C for 1.5-5 min. The PCR was completed by a final extension at 72°C for 5 min. The PCR products were purified with the Takara Agarose Gel DNA Purification Kit and used directly as templates for the cycle sequencing reactions. Sequence-specific primers were further designed and used as walking primers for both strands of each fragment with an ABI 3730 DNA sequencer (Applied Biosystems, USA).

The sequenced fragment was assembled into the mitochondrial genome using CodonCode Aligner (vers. 3) and BioEdit (vers. 7.0.1; Hall 1999). During the processing of large fragments and walking sequences, regular and manual examinations were made for reliable overlapping and assembly of the correct genomic sequence. Annotation and boundary determination of protein-coding genes and ribosomal (r)RNA genes were performed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov). Alignments with previously published mitogenomic sequences of flatfishes and other closely related bony fishes were carried out to ensure accuracy (Table 2). tRNA genes and their secondary cloverleaf structures were identified using tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE; Lowe and Eddy 1997), with cutoff values set to 1 when necessary. The *tRNA-Ser* secondary structure was further constructed by an RNA structure (Reuter and Mathews 2010) and confirmed by examination of the anticodon. A gene map of the *P. maxima* mitogenome was generated using CGView (Stothard and Wishart 2005). Base compositions of mitogenomic sequences were computed using MEGA 4.0 (Tamura et al. 2007).

Sequence analysis

Mitogenomes from 40 species were retrieved from GenBank, including 9 flatfishes

Table 1. Sequence information and location of DNA primers used in the polymerase chain reaction

Forward	Sequence (5'-3')	Location[a]	Tm (°C)
Pleur-Z15	ATTAAAGCATAACHCTGAAGATGTTAAGAT	15	48.8
Pleur-z2625	GTTTACGACCTCGATGTTGGATCAGGACAT	2568	56.5
Pleur-Z2510	GACCACTTTGATAGAGTG	3874	40.6
Pleur-Z4789	GTGCCCAAGCCAGCGAGC	5401	52.0
L-CO	CGTTCACTGGTTCCCCCTG	6779	50.3
L-CO3	CCCTACAATGGTTATCCTCTTC	8809	47.9
Pleur-Z8350	TTYGAAGCAGCCGCMTGATACTGACAYTT	9650	55.7
L-NDS	TCGGCTCAAAAACTTGTGGT	10,287	44.6
Pleur-Z9550	GAAGCTACCCTTATCCCAAC	10,985	46.7
L-FIN	GCTCGCAGCAATGAGGACT	12,074	48.1
Pleur-Z12420	TAAGGACGCCATCATTGAGGCC	13,403	51.6
Pleur-Z17054	GYCGGTGGTTARAATCCTCCCTACTGCT	15,828	57.8
Reverse	**Sequence (5'-3')**	**Location[a]**	**Tm (°C)**
Pleur-F2753	TAGATAGAAACTGACCTGGATTACTCCGGT	2668	55.2
H-CLU	GGAAGTGGTGTAGAGGAAGC	3990	48.7
Pleur-F6746	GCGGTGGATTGTAGACCCATARACAGAGGT	5578	58.6
Pleur-F5746	CCGAGGAATATTACTCCGAAATGG	6859	50.6
H-CO	GGGTGTGCTTGATGGGCCA	8939	50.3
Pleur-F11089	TTTAACCAAGACCRGGTGATTGGAAGTC	9893	54.1
H-CO3	ACAAGAGGTGGGAGCGATGA	10,408	48.7
Pleur-F9324	GTCAACTAGAAGAGGGAG	11,107	42.9
H-NDS	TAGCTGCTACTTGGATTTGC	12,213	44.6
H-FIN	AATGCGGAAACTGTAGATGGC	13,508	47.3
H-CY	GACGGACACCGGTCTAAATGG	16,012	51.2
H-DL	GAGGGTGAGGTTTAACGGGGG	658	53.1

Primers were used for the *Psetta maxima* specimen (no. EU419747). H and L refer to the heavy and light strands, respectively.
[a]Primer position within the *P. maxima* mitochondrial genome.

Table 2. Information of species used in this study

Classification[a]	Species	Accession no.	Reference
Clupeiformes			
Clupeidae	*Clupea pallasii*	AP009134	Lavoue et al. 2007
Beryciformes			
Berycidae	*Beryx decadactylus*	NC_004393	Miya et al. 2003
	Beryx splendens	NC_003188	Miya et al. 2001
Zeiformes			
Zeidae	*Zenopsis nebulosus*	NC_003173	Miya et al. 2001
	Zeus faber	NC_003190	Miya et al. 2001
Scorpaeniformes			
Dactylopteridae	*Dactyloptena peterseni*	NC_003194	Miya et al. 2001
	Dactyloptena tiltoni	NC_004402	Miya et al. 2003
Scorpaenidae	*Helicolenus hilgendorfi*	NC_003195	Miya et al. 2001
Cyclopteridae	*Aptocyclus ventricosus*	NC_008129	Miya et al. 2003
Cottidae	*Cottus reinii*	NC_004404	Miya et al. 2003
Perciformes			
Carangidae	*Carangoides armatus*	NC_004405	Miya et al. 2003
	Caranx melampygus	NC_004406	Miya et al. 2003
Emmelichthyidae	*Emmelichthys struhsakeri*	NC_004407	Miya et al. 2003
Sparidae	*Pagellus bogaraveo*	AB305023	Ponce et al. 2008
	Pagrus major	NC_003196	Miya et al. 2001
Chaetodontidae	*Chaetodon auripes*	AP006004	Yamanoue et al. 2007
Pomacanthidae	*Centropyge loriculus*	AP006006	Yamanoue et al. 2007
	Chaetodontoplus septentrionalis	AP006007	Yamanoue et al. 2007
Cichlidae	*Astronotus ocellatus*	AP009127	Mabuchi et al. 2007
Embiotocidae	*Ditrema temminckii*	AP009129	Mabuchi et al. 2007
Labridae	*Pseudolabrus sieboldi*	AP006019	Mabuchi et al. 2007
Blenniidae	*Salarias fasciatus*	NC_004412	Miya et al. 2003
Gobiesocidae	*Aspasma minima*	NC_008130	Miya et al. 2003
Rhyacichthyidae	*Rhyacichthys aspro*	NC_004414	Miya et al. 2003
Eleotridae	*Eleotris acanthopoma*	NC_004415	Miya et al. 2003
Acanthuridae	*Zebrasoma flavescens*	AP006032	Yamanoue et al. 2007
Scombridae	*Thunnus orientalis*	NC_008455	Takashima et al. 2006
	Auxis thazard	NC_005318	Catanese et al. 2008
Caproidae	*Antigonia capros*	AP002943	Miya et al. 2001
Pleuronectiformes			
Scophthalmidae	*Psetta maxima*	EU419747	This study
Paralichthyidae	*Paralichthys olivaceus*	AB028664	Saitoh et al. 2000
Pleuronectidae	*Kareius bicoloratus*	NC_003176	Miya et al. 2001
	Hippoglossus hippoglossus	NC_009709	Mjelle et al. 2008
	Hippoglossus stenolepis	NC_009710	Mjelle et al. 2008
	Reinhardtius hippoglossoides	NC_009711	Mjelle et al. 2008
	Verasper moseri	NC_008461	He et al. 2008
	Verasper variegatus	NC_007939	He et al. 2008
	Platichthys stellatus	NC_010966	Unpublished
Soleidae	*Solea senegalensis*	AB270760	Manchado et al. 2007
Tetraodontiformes			
Tetraodontidae	*Tetraodon nigroviridis*	AP006046	Yamanoue et al. 2006
Triodontidae	*Triodon macropterus*	AP009170	Yamanoue et al. 2007

[a]Classification followed Nelson (2006).

and 31 species representing 6 orders that are the potential sister-group to the Pleuronectiformes (Table 2). Ambiguous alignments of the control region, 2 rRNAs (12S and 16S), and the *ND6* gene were excluded from the phylogenetic analyses. The *ND6* gene was not used because of its heterogeneous base composition and consistently poor phylogenetic performance (Miya et al. 2003 2005, Peng et al. 2006). Eight tRNA genes encoded by the L-strand were converted into complementary strand sequences. A comparison of the phylogenetic performances between protein-coding and tRNA genes was performed. The *COI* gene was selected as a protein-coding gene reference marker (mainly at the family level), because of its similar length to the 22 concatenated tRNA genes and its popularity as an mtDNA marker in phylogenetic studies (Nylander et al. 1999, Hsu et al. 2009, Lu et al. 2009, Sharina and Kartavtsev 2010). The 22 concatenated tRNA genes were divided into 2 partitions: the stem (paired) and unpaired regions (including the bulge, loop, and unstructured single strand). Subsequently, 4 datasets (datasets 1-4, Table 3) were generated for the Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and maximum-likelihood (ML) analysis from 10 flatfishes and 2 outgroups.

In order to explore the sister-group to the Pleuronectiformes and the utility of the unpaired region of the tRNA gene, 2 other datasets were established for BI and ML analyses; the datasets consisted of 12 CDSs plus the stem region or complete tRNA sequence from 41 teleost species (datasets 5 and 6, Table 3).

The aligned sequences of the 2 tRNA regions contained only a few ambiguous alignments; therefore, we did not employ Gblock (Talavera and Castresana 2007) to eliminate those sequences. Two methods were used to test for substitution saturation using DMABE (Xia and Xie 2001), including substitution vs. the Tamura-Nei (TN93) genetic distance in pairwise comparisons and an entropy-based index (Xia et al. 2003) for the 1st, 2nd, and 3rd codon positions of the concatenated CDSs, the *COI* gene, and the stem and unpaired regions of the 41 fish mitogenomes. Chi-squared tests for the homogeneity of base frequencies and g1 statistics for all datasets (Table 3) were calculated using PAUP 4b10 (Swofford 1993).

ML analyses were implemented in PHYML (Guindon and Gascuel 2003). The best-fit evolutionary models for the ML analyses were determined using Modeltest 3.7 (Posada and Crandall 1998) from 56 models based on the Akaike information criterion (AIC). The GTR substitution model, fixed proportion of invariable sites, and gamma shape parameter were set, and results were evaluated with 300 bootstrap replicates. The generated trees were displayed using MEGA 4.0.

For the BI analyses, the best-fit models of nucleotide substitution for the 6 datasets were selected under different partitioning strategies with MrModeltest 2.1 (Nylander et al. 1999) (Table 3). Bayesian phylogenetic analyses were performed using general Lset values (e.g., nst and rates) and allowing the program to converge on the best estimates of the model parameters. Other parameter settings were as follows: each Markov chain was started from a random tree and run for (1.0-3.0) × 10^6 generations with every 100th generation sampled from the chain to assure the independence of the samples. Four chains, 3 heated (temperature = 0.5) and 1 cold, were simultaneously run using Metropolis-coupled

Table 3. Composition of different datasets and phylogenetic information evaluations

No.	Dataset[a]	g1 result	Heterogeneity test	Model selected
1	12, COI genes	-0.62	χ^2 = 24.48, p = 0.86	HKY+I+G
2	12, tRNA stems	-0.75	χ^2 = 7.59, p = 1.00	GTR+G
3	12, tRNA unpaired	-0.93	χ^2 = 14.61, p = 1.00	GTR+G
4	12, complete tRNA	-0.84	χ^2 = 12.10, p = 1.00	GTR+G[b]
5	41, CDSs and tRNA stems	-0.80	χ^2 = 122.15, p = 0.43	GTR+I+G[b]
6	41, CDSs and complete tRNA	-0.87	χ^2 = 131.18, p = 0.23	GTR+I+G[b]

[a]Datasets 1-4 were aligned based on the *COI* gene (1), the stem (2), and unpaired (3) and complete transfer (t)RNA sequences (4) of 22 concatenated tRNA genes from 12 fishes; datasets 5 and 6 were comprised of 12 concatenated protein-coding sequences (CDSs; excluding *ND6*) plus the stem regions or complete tRNAs of 22 concatenated tRNA sequences from 41 species in table 2. [b]The best-fit evolutionary model of datasets 4-6 were combined with different partitions, and the selected best-fit models were the same in different partitions, so only 1 model is provided.

Markov chain Monte Carlo (MCMC) to enhance the mixing capabilities of the Markov chains. In order to examine whether stationarity had been reached, fluctuating values of likelihood and all phylogenetic parameters were monitored graphically, and a simulation analysis was performed 3 times beginning from different random trees. To verify topological convergence among chains, "cumulative" diagnostic tools were implemented in AWTY (Nylander et al. 2008) with default settings. All sample points prior to stationarity were discarded as "burn in". Posterior probabilities for individual clades obtained from the 3 separate analyses were combined and summarized on a 50% majority-rule consensus tree for 6 datasets.

To evaluate the topological performances, site-wise log-likelihoods for each topology were calculated in PAUP 4.0b10, then 5 tests were implemented in CONSEL (Shimodaira and Hasegawa 2001), including the approximately unbiased (AU) test using the multiscale bootstrap technique (Shimodaira 2002), the weighted (wKH) and unweighted Kishino-Hasegawa tests (KH; Kishino and Hasegawa 1989), and weighted (wSH) and unweighted Shimodaira-Hasegawa test (SH; Shimodaira and Hasegawa 1999). Partitioned Bremer support (Baker and DeSalle 1997) was calculated using TreeRoot vers. 3 (Sorenson and Franzosa 2007) to assess the relative contribution of each gene or region to the Bayes topology derived from dataset 6 from 41 fish mitogenomes.

RESULTS

The complete mitogenome of *Psetta maxima*

Genomic organization

The complete mitochondrial sequence of *P. maxima* was 17,583 bp long (accession no. EU419747), including 13 protein-coding genes, 2 rRNA genes (*srRNA* and *lrRNA*), 22 tRNA genes, and a putative control region. Most of these genes are encoded on the H-strand, except the *ND6* and 8 tRNA genes, which are encoded on the L-strand (Table 4, Fig. 1). Overall base composition values of the L-strand mtDNA were 27%, 27%, 17%, and 29% for A, C, G, and T, respectively, with an A+T content of 56%. Thirteen intergenic spacers were present ranging from 1 to 6 bp except for 2 unusual spacers between *ND2* and *tRNA-Trp* (41 bp), and *COIII* and *tRNA-Gly* (132 bp). Seven overlapping areas were observed, including 4 notable overlapping positions between protein-coding genes (*ATP8* and *ATP6*, *ATP6* and *COIII*, *ND4L* and *ND4*, and *ND5* and *ND6*), which were reported in other fish and vertebrate species (Table 4, Fig. 1).

Protein-coding genes, rRNA genes, and non-coding regions

All of the 13 protein-coding genes in the *P. maxima* mitogenome were organized in a similar manner as those in other fishes. Twelve of the 13 genes began with an ATG start codon, with the exception of the *COI* gene (GTG). As in other fishes, *P. maxima* mtDNA also uses TAA, TAG, or T as stop codons. A striking finding in this study was the existence of an 1100-bp-long *ND2* gene, which is the longest one to date found in over 700 teleost mitogenomes. The normal size of *ND2* in flatfishes is approximately 1043 ± 3 bp. The 696-bp *ATP6* gene was also the longest one in flatfishes (683 or 684 bp). The sizes of other protein-coding genes were highly conserved across species in the Pleuronectiformes compared to corresponding data (Kong et al. 2009) (Table 4, Fig. 1).

Due to length variations of the 2 genes and the 132-bp intergenic spacer in *P. maxima*, 6 fragments in 2 more individuals from different locations were sequenced for further confirmation (GenBank accession nos. HM562729-HM562734). The results indicated that the unusual lengths were stable characteristics among individuals in the present study. Comparisons of *ND2* amino acid (aa) sequences between *P. maxima* (370 aa) and normal type *Paralichthys olivaceus* (349 aa) demonstrated that the *ND2* gene of *P. maxima* was more variable in the last 40 aa (including 21 longer aa). Two important protein domains (Oxidored_q1 and NADH_dehy_S2_C) detected by the SMART website server (http://smart.embl-heidelberg.de; Letunic et al. 2009) were similar to those in *P. olivaceus*.

12S *srRNA* and 16S *lrRNA* were 947 and 1712 bp long, respectively, and located regularly between *tRNA-Phe* and *tRNA-Leu*, and separated by *tRNA-Val* (Table 4, Fig. 1). The 1658-bp putative control region (CR) was commonly situated between the *tRNA-Pro* and *tRNA-Phe* genes. An 804-bp-long repeat array at the 3' end of the CR region was observed, which is a common feature in the mitogenomes of flatfishes and bony fishes. The array was composed of 2 subarrays, a 12-bp motif with 61.4 repeats and an 11-bp motif with 6.4 repeats (Table 4, Fig. 1).

tRNA genes

The sizes of the 22 tRNA genes varied from 67 (*tRNA-Cys*) to 74 bp (*tRNA-Leu* and *tRNA-Lys*), and the total length was 1551 bp. Twenty-one tRNA genes and their secondary structure were recognized by tRNAscan-SE, while *tRNA-Ser* was identified by comparison to other flatfishes. The secondary structure of *tRNA-Ser* was predicted via its RNA structure, followed by a manual examination (Fig. 2). The DHU-arm had only a 3-base D-loop and a 3-pair base stem (T=A, C≡G, and G≡C). The 3-pair base stem was also observed in other tRNA genes, such as *tRNA- Ser* (*UGA*), *Val*, and *Tyr*, while the 3-base D-loop was rarely found. Usually, the secondary structure of *tRNA-Ser* only possesses a loop without a stem or occasionally a stem without a loop. Likely for this reason, the DHU-arm was commonly believed to have been lost in many fishes (Lee and Kocher 1995, Miya and Nishida 1999). The overall base contents of the stem and unpaired regions of the 22 concatenated tRNA genes were computed. In unpaired regions, the A+T content (66.0%) was much higher than that of C+G (34.0%), with a strong bias favoring “A”. In contrast, the C+G

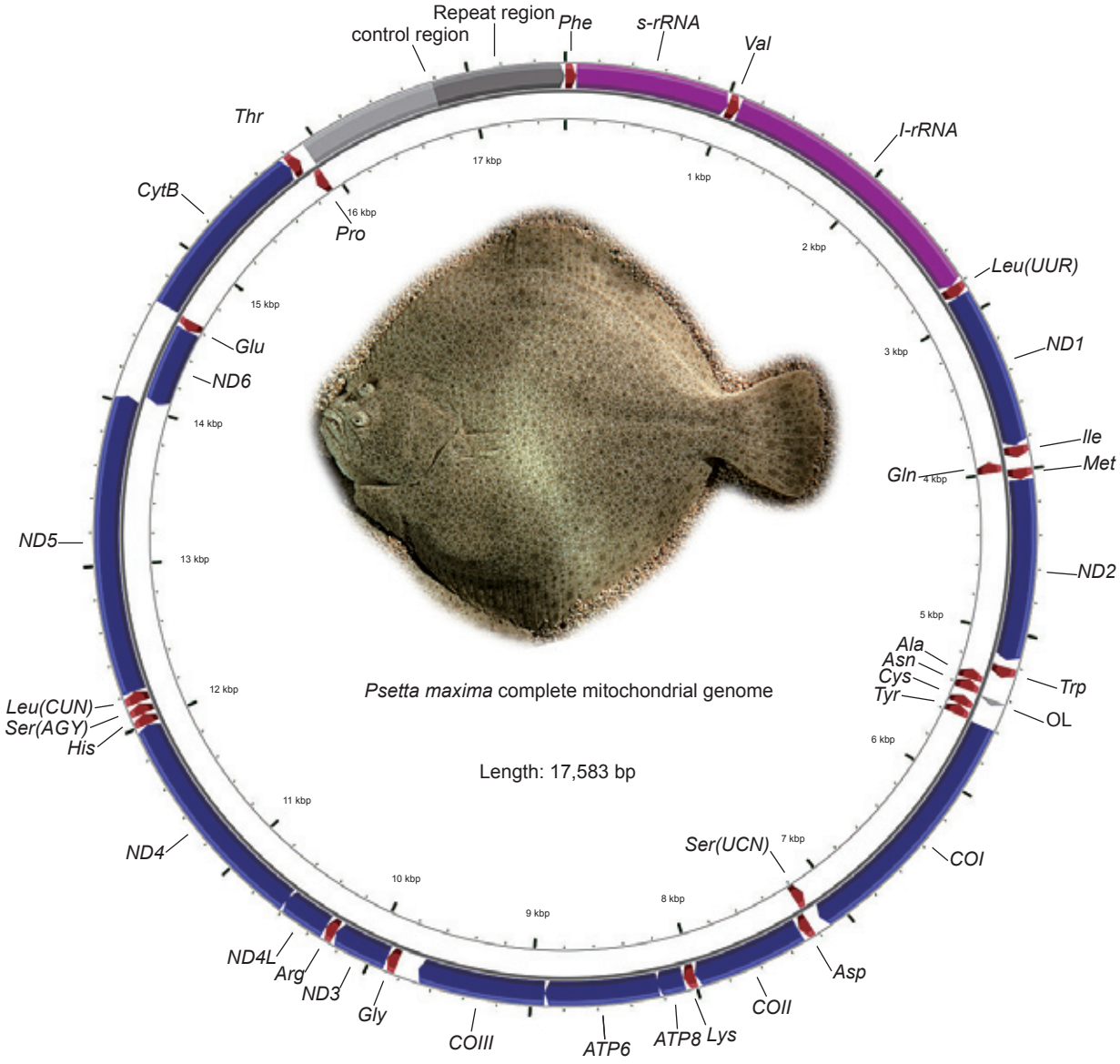

Fig. 1. Gene map of the mitochondrial genome of *Psetta maxima* (Photo by Luc Viatour).

content (56.0%) in stem regions was higher than that of A+T (44.0%). The base compositions of the other 9 flatfishes showed the same trend as those of *P. maxima* (Table 5). These data provide evidence that the tempo and mode of sequence evolution differed between the stem and unpaired regions of the tRNA gene (Kumazawa and Nishida 1993).

Phylogenetic information evaluation of different datasets

The substitution saturation tests for the 1st, 2nd, and 3rd codon positions of the CDSs and *COI*, and the tRNA datasets (stem and unpaired region individually) demonstrated that the pairwise transition (TS) and transversion (TV) differences

Table 4. Organization of the *Psetta maxima* mitochondrial genome

Name	Length (bp)	Start-End	Strand	Start/stop codon and anticodon	Intergenic region[a]
Phe	68	1-68	H	UUC	0
12S	945	69-1013	H		0
Val	72	1014-1085	H	GUA	1
16S	1712	1087-2798	H		0
Leu(UUA)	74	2799-2872	H	UUA	0
ND1	975	2873-3847	H	ATG/TAG	4
Ile	71	3852-3922	H	AUC	-1
Gln	71	3922-3992	L	CAA	-1
Met	69	3992-4060	H	AUG	1
ND2	1110	4062-5171	H	ATG/TAA	41
Trp	71	5213-5283	H	UGA	1
Ala	69	5285-5353	L	GCA	1
Asn	73	5355-5427	L	AAC	0
OL	38	5428-5465	H		-3
Cys	67	5463-5529	L	UGC	0
Tyr	70	5530-5599	L	UAC	1
COI	1551	5601-7151	H	GTG/TAA	0
Ser(UCA)	71	7152-7222	L	UCA	3
Asp	69	7226-7294	H	GAC	6
COII	691	7301-7991	H	ATG/T	0
Lys	74	7992-8065	H	AAA	1
ATP8	168	8067-8234	H	ATA/TAA	-10
ATP6	696	8225-8920	H	ATG/TAA	-1
COIII	786	8920-9705	H	ATG/TAA	132
Gly	71	9838-9908	H	GGA	0
ND3	351	9909-10,259	H	ATG/TAG	-2
Arg	69	10,258-10,326	H	CGA	0
ND4L	297	10,327-10,623	H	ATG/TAA	-7
ND4	1381	10,617-11,997	H	ATG/T	0
His	69	11,998-12,066	H	CAC	0
Ser(AGC)	68	12,067-12,134	H	AGC	5
Leu(CUA)	73	12,140-12,212	H	CUA	0
ND5	1839	12,213-14,051	H	ATG/TAA	-4
ND6	522	14,048-14,569	L	ATG/TAA	0
Glu	69	14,570-14,638	L	GAA	4
CytB	1141	14,643-15,783	H	ATG/T	0
Thr	72	15,784-15,855	H	ACA	-1
Pro	71	15,855-15,925	L	CCA	0
D-loop	1658	15,926-17,583	H		
repeat region	801	16,779-17,579	H		

[a]The intergenic region refers to noncoding bases between the feature on the same line and the feature on the line below, with a negative number indicating an overlap.

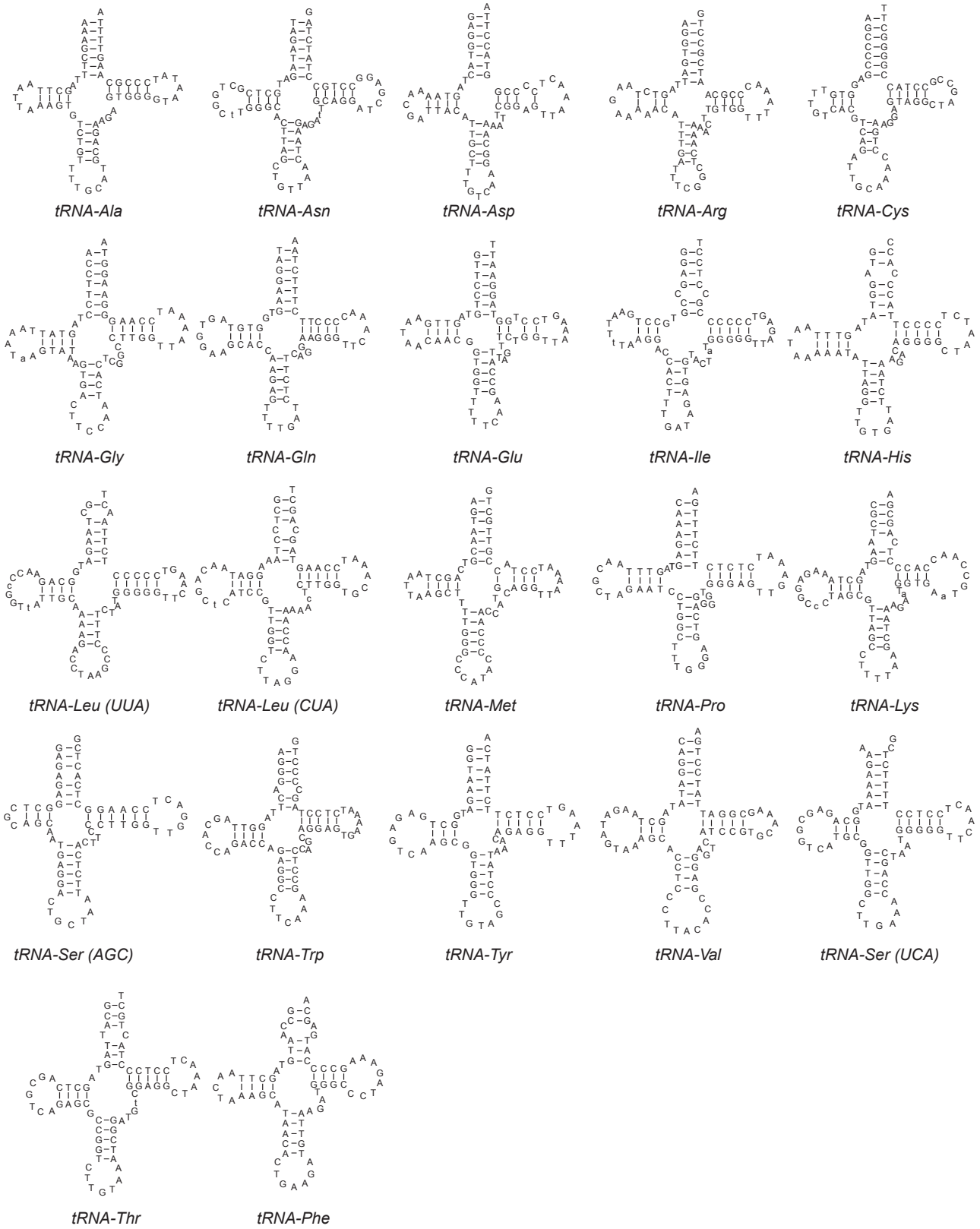

Fig. 2. Predicted cloverleaf structures of 22 transfer (t)RNAs from the mitogenome of *P. maxima*.

increased over increasing evolutionary distances. The only exception was TS differences at the 3rd codon position of the CDSs, in which a small amount of saturation was detected; no saturation was detected for the other sequences (Fig. 3). These results were consistent with the entropy-based index of substitution saturation, and substitution saturation at the 3rd codon positions of CDSs was revealed by remarkably greater values of *Iss* compared to those of *Iss.c* (Table 6). Accordingly, when phylogenetic analyses were carried out, the 3rd codon positions of CDSs were excluded. The g1 statistics of the 6 datasets (from -0.62 to -0.87), which were significant according to Hillis and Huelsenbeck (1992), suggested that the datasets contained useful phylogenetic signals. The heterogeneity test showed no heterogeneity in these datasets (Table 3).

Utility of tRNA genes for phylogenetic analyses

Comparisons of phylogenetic performances of the ML and BI analyses between tRNA and *COI* genes were carried out based on the 4 datasets of the *COI* gene, the stem, unpaired regions, and entire tRNA gene of 22 concatenated tRNA sequences from 10 flatfishes with *C. pallasii* (Clupeiformes) and *Z. nebulosus* (Zeiformes) as outgroups (Table 3). Due to the similar topologies and less information at some branches in the ML analyses, we mainly discuss the results of the BI analyses.

In the BI topologies derived from the *COI* and stem regions within the Pleuronectidae, species of *Platichthys* and *Kareius* formed a clade, and then sister taxa joined to other Pleuronectidae fishes, supported by 100% Bayesian posterior

Table 5. Base compositions of different regions of 22 concatenated transfer (t)RNA from 10 flatfishes

Species	Unpaired regions				Stem regions				Complete sequences			
	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)
Psetta maxima	36.9	17.4	16.7	29.1	20.1	26	30	23.9	27.9	21.9	23.9	26.4
Paralichthys olivaceus	37.4	16.8	16.9	28.8	21.1	26.2	28.9	23.9	28	21.8	23.7	26.5
Kareius bicoloratus	37.9	16.3	16.3	29.4	20.4	25.9	29.5	24.2	27.8	21.9	23.9	26.4
Hippoglossus hippoglossus	37.7	16.1	16.6	29.6	20.4	26	29.5	24.1	28.2	21.8	23.5	26.6
H. stenolepis	37.6	16.3	16.8	29.3	20.5	25.9	29.2	24.3	27.7	21.5	24.1	26.7
Reinhardtius hippoglossoides	37.4	16.5	17	29.2	20.6	25.9	29.2	24.2	27.7	21.8	23.9	26.6
Verasper moseri	37.3	15.4	17.3	30	20.3	26.4	29.2	24.1	27.4	22.2	24.2	26.2
V. variegatus	37.8	15.8	16.7	29.7	20	26.4	29.5	24	28.3	21.4	23.6	26.7
Platichthys stellatus	36.6	15.8	18.1	29.6	20.3	26.1	29.4	24.1	27.9	21.7	23.8	26.6
Solea senegalensis	37.9	14.3	16	31.8	20.7	26.8	29.7	22.8	27.8	21.7	24.1	26.4

Table 6. Entropy-based index of substitution saturation of different partitions for 41 teleostean mitochondrial DNA genomes

			For a symmetrical tree			For an extremely asymmetrical tree		
	Proportion of invariable sites	*Iss*	*Iss.c*	*d.f.*	*p*	*Iss.c*	*d.f.*	*p*
CDS-1st	0.39544	0.369	0.848	2230	0	0.837	2230	0
CDS-2nd	0.01749	0.106	0.848	3622	0	0.837	3622	0
CDS-3rd	0.00564	1.356	0.859	10,214	0	0.929	10,214	0
tRNA-stem	0.00373	0.164	0.814	871	0	0.781	871	0
tRNA-Unpaired	0.00517	0.360	0.809	749	0	0.775	749	0
COI	0.06788	0.266	0.834	1427	0	0.804	1427	0

Values of *Iss* remarkably or unremarkably smaller than that of *Iss.c* indicate slight saturation or saturation. Values of *Iss* remarkably or unremarkably bigger than that of *Iss.c* mean the data are useless or almost useless for phylogenetic analyses. CDS, concatenated protein-coding sequence.

probabilities (Fig. 4A, B), which were consistent with the hypotheses of previous results (Li and Wang 1995, Cooper and Chapleau 1998, Roje 2010).

The relationship between *Psetta maxima* and *Solea senegalensis* inferred from the *COI* gene was not well resolved (Fig. 4A), and these 2 species were clustered as a sister group in the tree generated by the stem dataset (Fig. 4B). In the 2 other trees derived from unpaired regions and complete tRNA genes (Fig. 4C, D), *P. maxima* was a sister taxon to all Pleuronectidae species, including species of the Paralichthyidae and Pleuronectidae (Pleuronectoidei).

Sister group relationship to the Pleuronectiformes

To explore the sister group relationship to the Pleuronectiformes, 6 orders which are the potential sister-group to flatfishes were selected. Four phylogenetic trees were constructed using the BI and ML analyses based on datasets 5 and 6 from 41 teleost mitogenomes with *C. pallasii* (Clupeiformes) as an outgroup (Figs. 5, 6); thereafter, we evaluated the topological performances using the AU, KH, SH, wSH, and wKH tests. The results showed that the BI analyses yielded the 1st and 2nd best topologies (Table 7). Both topologies based on datasets 5 and 6 exhibited similar phylogenetic relationships as follows: (Clupeiformes, (Zeiformes, (Beryciformes, (partial Perciformes species, Pleuronectiformes)))) (Figs. 5, 6). Zeiformes and Beryciformes fishes first split off from the other 4 orders (Scorpaeniformes, Perciformes, Pleuronectiformes, and Tetraodontiformes), 10 flatfishes were clustered into a monophyletic group,

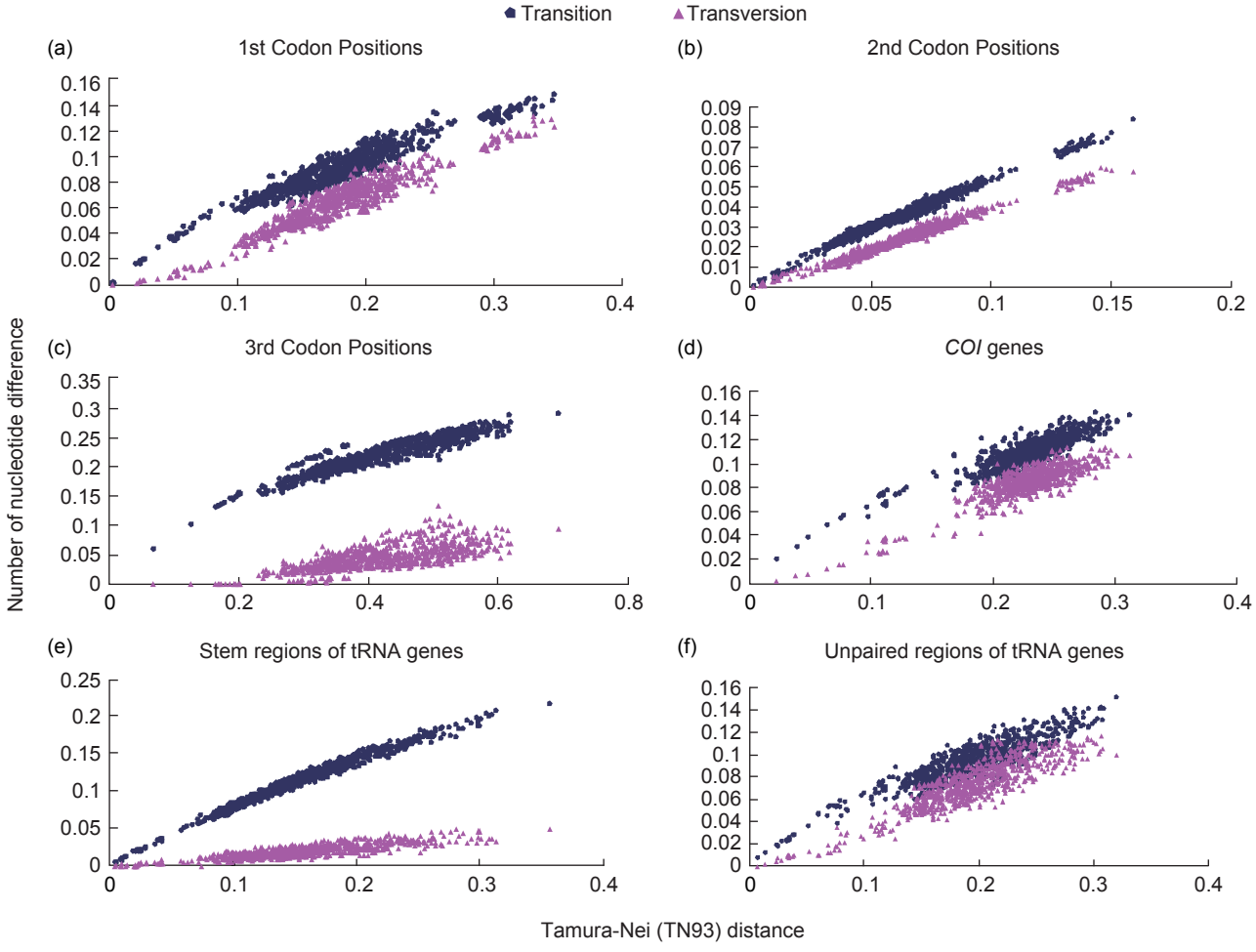

Fig. 3. Number of transition and transversion substitutions vs. the Tamura-Nei (TN93) distance in pairwise comparisons for the 1st (a), 2nd (b), and 3rd (c) codon positions of concatenated protein-coding sequences (CDSs), *COI* genes (d), and stem (e) and unpaired (f) regions of transfer (t)RNA genes from 41 teleostean mitochondrial (mt)DNA.

and Carangidae fishes were placed as sister taxa to the Pleuronectiformes. It should be noted that *P. maxima* and *S. senegalensis* were still clustered into 1 clade in the BI and ML trees inferred from dataset 5 (which only included stem region (Fig. 6A, B)), whereas, when the unpaired region was added (dataset 6), the topologies changed (Figs. 5, 6C).

Partitioned Bremer support values were also calculated for the BI topologies derived from dataset 6. Of all 15 partitions (12 CDSs, stem, and unpaired and complete tRNA), the highest value (115.00) was obtained from complete tRNA genes, followed by *ND5* (86.33), unpaired tRNA (70.67), *ND4* (52.67), and tRNA stem regions (44.33) (Table 8, Fig. 5). These values indicated that the stem and unpaired regions of the tRNA gene contributed significant signals (in total 26.4%) to the phylogenetic analyses.

DISCUSSION

Features of the mitochondrial genome of *Psetta maxima*

We determined the complete mitochondrial genome sequence of *P. maxima*, and the results

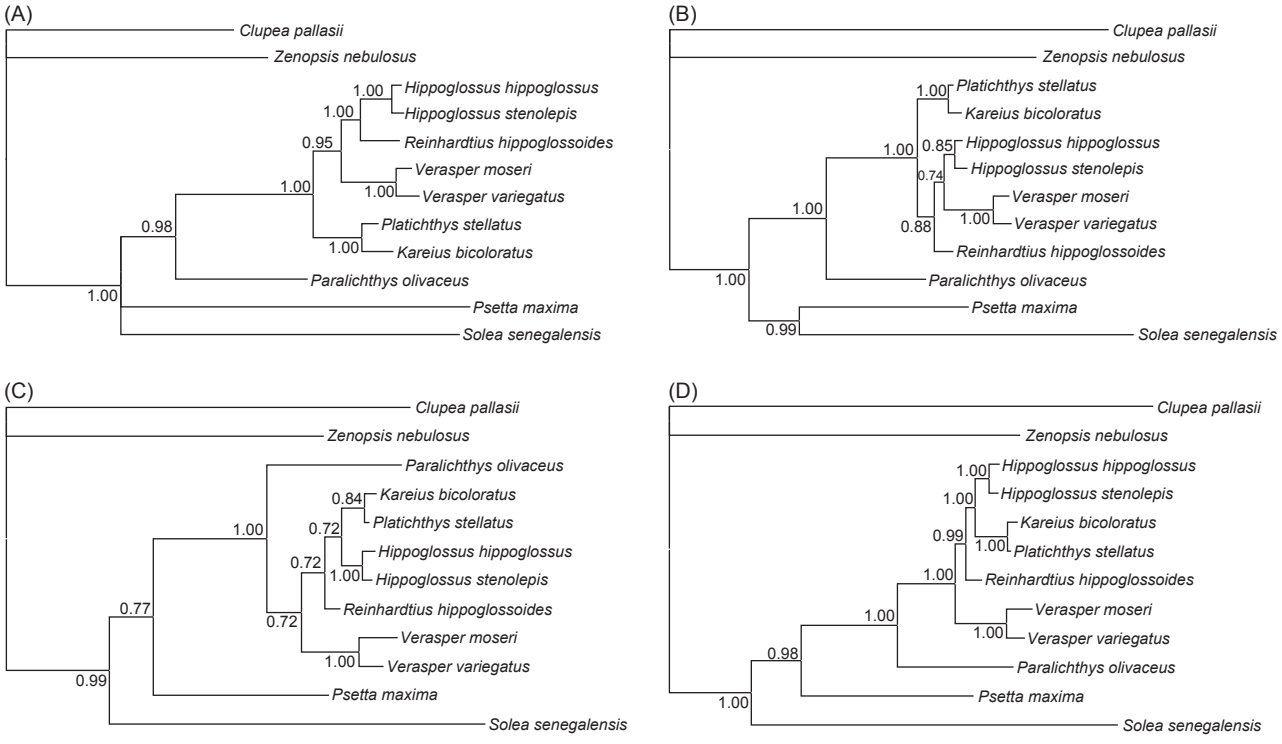

Fig. 4. The 50% majority rule consensus tree estimated from the partitioned Bayesian analysis based on *COI* genes (A), stem regions (B), unpaired regions (C), and entire (D) tRNA gene sequences of 10 flatfishes with *Clupea pallasii* and *Zenopsis nebulosus* as outgroups. Numbers beside the internal branches indicate Bayesian posterior probabilities.

Table 7. Values of 5 tests for 4 phylogenetic analyses

	Rank	AU	KH	SH	wKH	wSH
BI (dataset 5)	1	0.961	0.919	0.996	0.919	0.996
BI (dataset 6)	2	0.103	0.081	0.242	0.081	0.173
ML (dataset 5)	3	0.076	0.060	0.225	0.060	0.132
ML (dataset 6)	4	0.061	0.032	0.080	0.032	0.078

AU, approximately unbiased test; KH, Kishino-Hasegawa test; SH, Shimodaira-Hasegawa test; wKH, weighted Kishino-Hasegawa test; wSH, weighted Shimodaira-Hasegawa test; BI, Bayesian inference; ML, maximum-likelihood. ** $p < 0.01$.

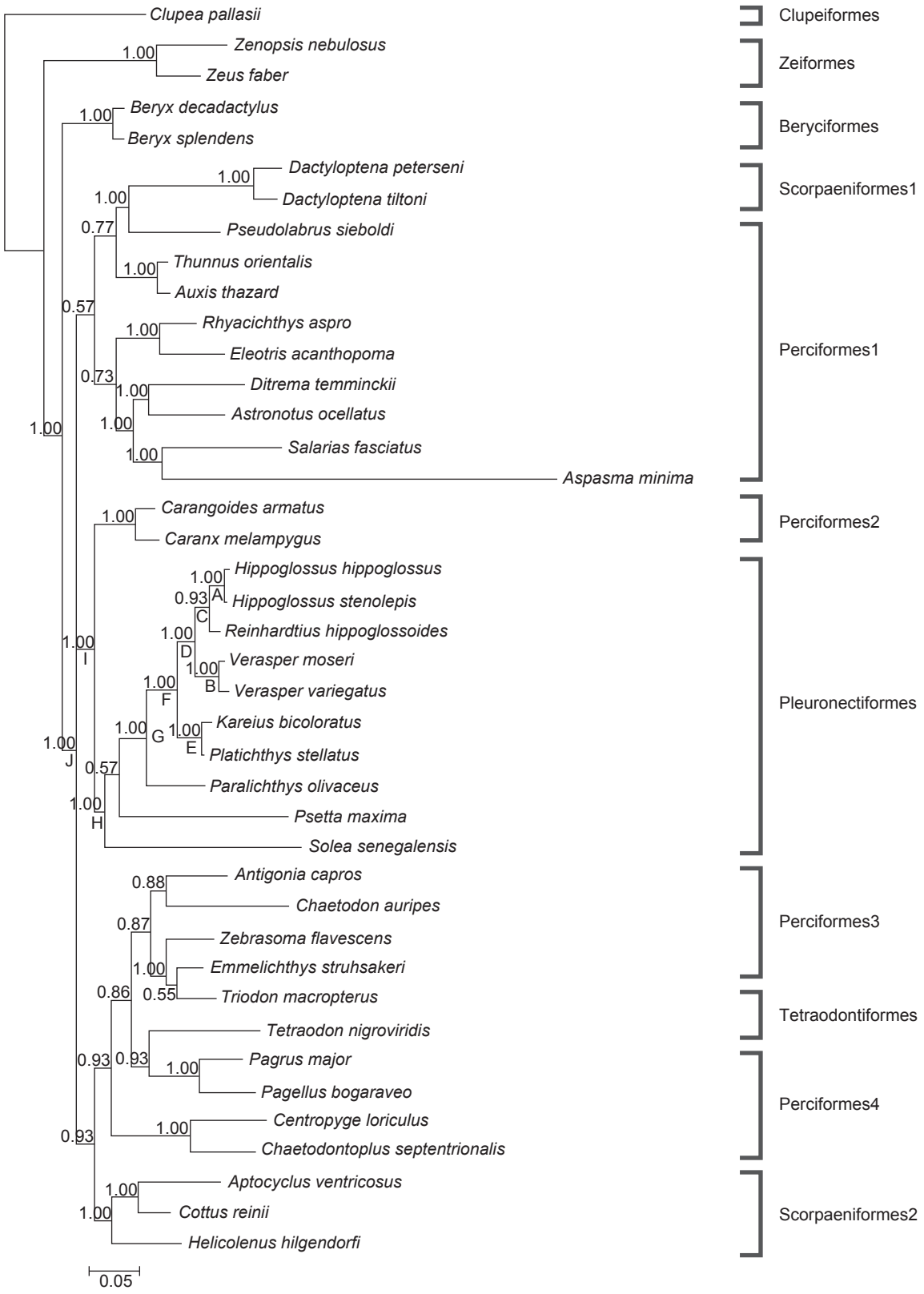

Fig. 5. The 50% majority rule consensus tree estimated from the partitioned Bayesian analysis derived from 41 teleost mitogenomes using the 1st and 2nd codon positions of 12 protein-coding genes and 22 tRNA genes sequences. Numbers beside the internal branches indicate Bayesian posterior probabilities. Uppercase letters exhibit the positions of the Bremer support values shown in table 8.

showed that this mitogenome is very similar to that of other teleosts with respect to the organization and composition, and a striking characteristic of this species was the novel lengths of *ND2* and the *ATP6* gene, both of which are the longest among 700 flatfish mitogenomes sequenced so far. These particular attributes may help the study of the evolution of mitogenomic constructors and exploring phylogenetic relationships of this species.

Utility of tRNA genes for phylogenic analyses

Currently, the numbers of fish mitogenomes are increasing rapidly and are widely being used for high-level phylogenetic analyses. However, sequences of tRNAs or especially unpaired regions have not received much attention to date for phylogenetic purposes. Generally, the reasons for not using tRNA or unpaired regions were presumably due to frequent length and point mutations, and their different modes and rates of evolution (Brown et al. 1982, Gadaleta et al. 1989, Miya et al. 2003 2005). To date, only Kumazawa and Nishida (1993) illustrated the possible usefulness of tRNA stem sequences in deep-branch analyses, but there were no further studies on whether unpaired regions of tRNA genes are applicable or not.

In the present study, comparisons were made among BI phylogenetic trees derived from datasets 1-6 (Table 3). The BI trees based on datasets 1-4 did not show large deviations to acknowledged relationships in the Pleuronectiformes. Among these, the COI tree did not resolve the relationship between *P. maxima* and *S. senegalensis* (Fig. 4A), and the stem tree displayed the 2 species clustered as a sister group (Fig. 4B); the other 2 trees based on unpaired regions and complete tRNA genes (Figs. 4C, D) showed *P. maxima* as a sister taxon to all Pleuronectidae species, and those results seem more reasonable than the COI and stem ones, which were consistent with other molecular and morphologic analyses (Li 1995, Chapleau 1993, Berendzen and Dimmick 2002).

The utility of the tRNA unpaired region was further supported by the BI and ML phylogenetic analyses based on datasets 5 and 6. The trees based on dataset 5 still displayed a sister-relationship between *P. maxima* and *S. senegalensis* (Figs. 6A, B). Interestingly, the relationship changed when the unpaired region was added (Figs. 5, 6C). Furthermore, the higher partitioned Bremer support values of the tRNA stem or unpaired regions (44.33 and 70.67) also provided evidence that both regions of tRNA gene sequences could contribute a great quantity of information to phylogenetic analyses (Table 8, Fig. 5).

Both the stem and unpaired regions of tRNA genes can provide some extent of useful and specific information for phylogenetic analyses. The reasons are summarized as follows: the analyses of g1, heterogeneity, and substitution saturation test proved that the stem and unpaired regions can be used in phylogenetic analyses. The topologies of the phylogenetic trees based on each region of the tRNA gene did not show large deviations to acknowledged relationships in the Pleuronectiformes, even when the entire CDS dataset was used. These results indicate the availability of tRNA sequences, and both regions could increase the amount of information and avoid directional migration of phylogenetic analyses in terms of using protein-coding genes alone. Furthermore, results of the partitioned Bremer data also supported the stem and unpaired regions being able to provide ample information for phylogenetic analyses. Therefore, we suggest that phylogenetic analyses can be more reasonable and objective if the 2 regions of tRNA genes are included.

In addition, due to the different evolutionary tempos and modes for each partition of datasets, the strategy of model selection for individual partitions should be taken into account, such as the stem or unpaired regions of tRNA and protein-coding genes in this study (Table 3).

Phylogenetic relationship of the Pleuronectiformes

Since the end of the last century, some pertinent studies on the sister-group to the Pleuronectiformes were conducted; however, the question has not been adequately answered so far. In this study, phylogenetic analyses based on 41 complete teleostean mitogenomes were used in an attempt to explore the relationship between the Pleuronectiformes and other teleosteans. The topologies suggested that the Pleuronectiformes as represented by 10 flatfishes is a monophylogenic group, which is consistent with previous results using morphological or molecular data (Chapleau 1993, Li and Wang 1995, Berendzen and Dimmick 2002, Pardo et al. 2005). The phylogenetic analyses also indicated that flatfishes are distantly related to Clupeiformes

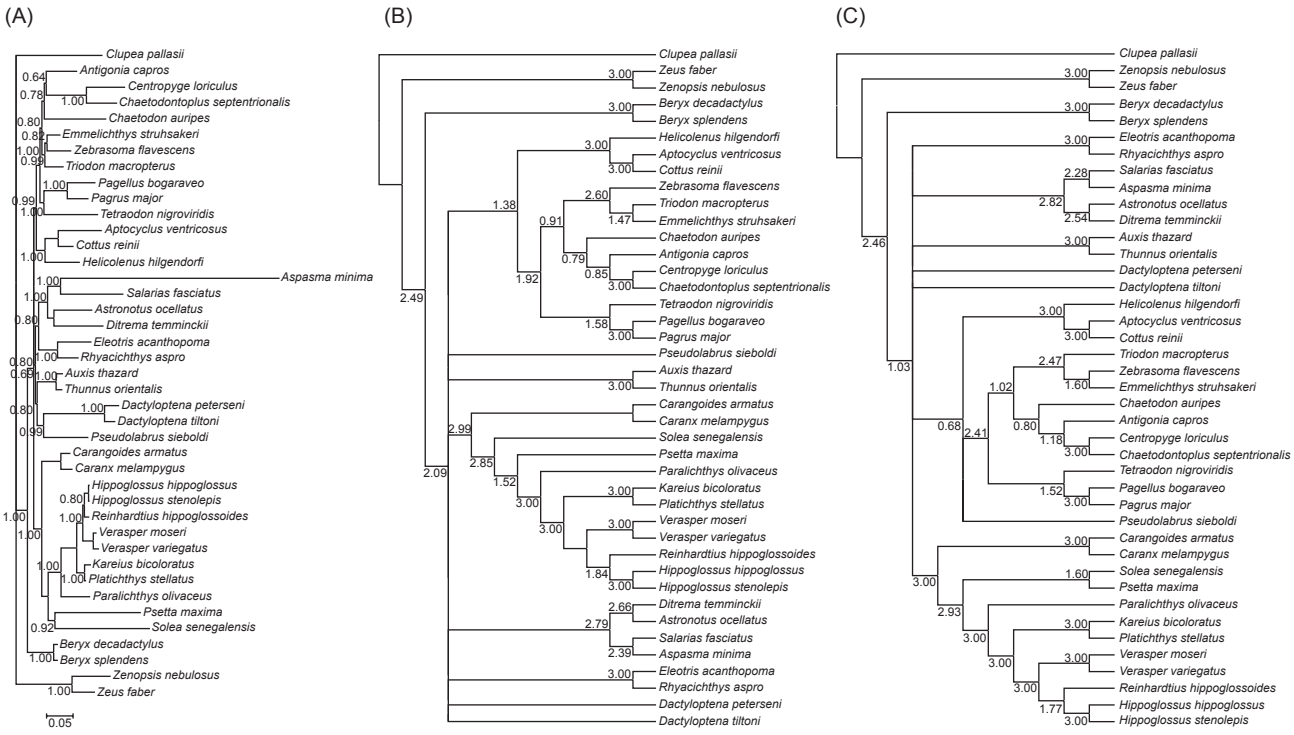

Fig. 6. (A) The 50% majority rule consensus tree estimated from the partitioned Bayesian analysis derived from 40 teleostean mitochondrial (mt)DNA with *Clupea pallasii* as the outgroup, including the 1st and 2nd codon positions of 12 protein-coding genes and stem regions of 22 tRNA genes. Numbers beside the internal branches indicate Bayesian posterior probabilities. (B) The 50% majority rule consensus maximum-likelihood (ML) tree derived from 40 teleostean mtDNA with *C. pallasii* as the outgroup, including the 1st and 2nd codon positions of 12 protein-coding genes and stem regions of 22 tRNA genes. Numbers beside the internal branches indicate bootstrap values. (C) The 50% majority rule consensus ML tree derived from 40 teleostean mtDNA genomes with *C. pallasii* as the outgroup, including the 1st and 2nd codon positions of 12 protein-coding genes and 22 tRNA genes. Numbers beside the internal branches indicate bootstrap values.

Table 8. Values of partitioned Bremer support for the Bayesian inference tree

Character\nodes[a]	A	B	C	D	E	F	G	H	I	J	Sum
ND3	2.00	4.00	0.00	2.00	0.00	3.00	1.00	-3.00	-8.50	0.00	0.50
ND4L	2.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	-1.00	3.00	5.00
ND1	2.00	1.00	1.00	4.00	1.00	7.00	0.67	0.00	-1.50	-9.00	6.17
ATP8	0.00	6.00	0.00	1.00	0.00	2.00	2.67	1.00	-1.00	0.00	11.67
CYTB	5.00	1.00	1.00	-1.00	7.00	8.00	4.00	5.00	-0.50	-13.00	16.50
COI	0.00	4.00	-1.00	1.00	1.00	7.00	0.67	2.00	4.00	-1.00	17.67
ND2	2.00	8.00	-1.00	-2.00	3.00	16.00	1.33	2.00	3.00	-7.00	25.33
COIII	0.00	4.00	4.00	-1.00	2.00	3.00	4.33	3.00	8.50	2.00	29.83
COII	2.00	2.00	-1.00	3.00	7.00	2.00	4.33	1.00	11.50	1.00	32.83
ATP6	2.00	12.00	1.00	4.00	6.00	1.00	3.00	1.00	2.50	3.00	35.50
Stem[b]	2.00	13.00	-1.00	1.00	7.00	12.00	3.33	-1.00	-2.00	10.00	44.33
ND4	7.00	9.00	3.00	3.00	11.00	14.00	5.67	0.00	0.00	0.00	52.67
Unpaired[b]	5.00	19.00	1.00	-3.00	7.00	4.00	14.67	4.00	1.00	18.00	70.67
ND5	13.00	17.00	1.00	12.00	9.00	11.00	14.33	4.00	4.00	1.00	86.33
Entire[b]	7.00	32.00	0.00	-2.00	14.00	16.00	18.00	3.00	-1.00	28.00	115.00

[a]Nodes correspond to uppercase letters in figure 5. ‘‘Sum” is the total Bremer support for each gene. [b]Different partitions of 22 transfer (t)RNA concatenated genes.

teleosts and are a sister-group to Carangidae species of the Perciformes (Fig. 5). These results are congruent with previous molecular hypotheses using mitochondrial and nuclear DNA genes (Miya et al. 2003 2005, Dettai and Lecointre 2005, Li et al. 2009, Little et al. 2010), and also favored the hypothesis of lower-percoid origins for flatfishes (Chapleau 1993). We also admitted that flatfishes and the Carangidae split phylogenetically from a common ancestor most recently, and these taxa did not show close evolutionary affinities with the Zeiformes, which was in contrast to findings of Li and Wang (1995) and Boulenger (1901), who believed that flatfish might have originated from the early Clupeoidei and dories during the Cretaceous period, respectively.

Due to the limited data of representative taxonomic sampling and molecular markers, the relationship between specific groups representing lower-percoids and Pleuronectiformes is still not clearly elucidated. Increasing the intensity of taxon sampling and using additional independent DNA characters should be conducted in the future to better define the molecular evolutionary origin and phylogenetic issues of flatfishes.

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