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# COMPARATIVE ANALYSIS OF 5'-END SEQUENCE OF THE MITOCHONDRIAL CONTROL REGION OF SIX FLATFISH SPECIES (PLEURONECTIDAE) FROM THE YELLOW SEA

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***ABSTRACT.* –** The 5'-end sequence of the mitochondrial control region (CR) of six flatfishes (Pleuronectidae: Teleostei) from the Yellow Sea (China) and one from the North Sea (Belgium) were determined using polymerase chain reaction (PCR) and direct sequencing. The seven species are the pointhead plaice (*Cleisthenes herzensteini*), stone flounder (*Kareius bicoloratus*), variegated flounder (*Verasper variegatus*), slime flounder (*Microstomus achne*), finespotted flounder (*Pleuronichthys cornutus*), yellow striped flounder (*Pseudopleuronectes herzensteini*) and common dab (*Limanda limanda*). The sequences obtained were compared and analyzed with those of 10 other flatfish taxa available in databases from the families Paralichthyidae, Pleuronectidae, Scophthalmidae and Soleidae. No tandem repeats were detected at the beginning of this CR sequence of the seven species studied. The structural arrangement of the sequence appeared highly similar among these species, despite their 5'-end portion being more variable and 3'-end portion more conserved. The terminal associated sequence (TAS) motif and its complementary motif (cTAS) were found at the 5'-end portion and the Extended Terminal Associated Sequence (ETAS) of flatfish and its potential secondary structure was described and analyzed. A conserved motif (CM5'd) was also identified in the 5'-end portion through comparisons with previous studies. Both complete and partial conserved sequence blocks (CSB-F and CSB-D) were detected in the central conserved region (CCR) domain. Molecular phylogenetic analysis of the 17 species was conducted based on the 5'-end CR sequence. The phylogenetic relationships agreed well with those based on morphology at the family level and most of those at the genus level. Ten genera of Pleuronectidae grouped firmly in a big branch in the phylogenetic tree generated, supporting their monophyletic origin. Large intra-generic distances between two *Limanda* species supported the homoplasy observed in the genus *Limanda*. However, the discrepancy in relationship of *Pleuronichthys cornutus* to all other species at the molecular and morphological levels in Pleuronectidae, suggested the need for further phylogenetic analysis. The results in this study indicated that 5'-end CR sequence could be reliably used for the analysis and testing of phylogenetic relationships within the Pleuronectiformes.

***KEY WORDS.* –** Pleuronectidae, mitochondrial control region, sequence, TAS, ETAS, CSB.

## INTRODUCTION

The Pleuronectiformes or flatfishes are one of the most species-rich groups of fish in the world. There are approximately 572 extant species in this group representing 123 genera in14 families (Chapleau, 1993; Nelson, 1994). About 134 species (50 genera in 8 families) have been recorded from seas surrounding the coast of China, with 19 species from the family Pleuronectidae (Li & Wang, 1995). While the framework of conventional phylogeny and systematics was built based on morphological characters, DNA sequence data have been used extensively in the determination, reconstruction of phylogenetic relationships and evolutionary studies at molecular level for the last decade (Avise, 1994; Lee et al., 1995; Kocher & Stepien, 1997; Stepien, 1999; Tinti et al., 1999, 2000; Berendzen & Dimmick, 2002).

With the ease of obtaining sequence information by selective fragment amplification with universally-conserved primers and its intrinsic features, mitochondrial DNA (mtDNA) sequences have been widely used as an important and

convenient molecular marker for studies of genetics, evolution, phylogeny and molecular ecology (Brown et al., 1979; Harrison, 1989; Avise, 1994; Lee et al., 1995). Besides sequences coding for proteins, tRNAs and rRNAs, vertebrate mtDNA has a single non-coding segment called the control region (CR). The CR contains the H- and L-strand promoters (LSP and HSP) for transcription, the H-strand replication origin (OH) and the displacement loop (D-loop) (Chang & Clayton, 1986; Shadel & Clayton, 1997). The vertebrate mtDNA CR usually consists of a central conserved sequence portion surrounded by two highly variable, adenine-rich domains (Kocher et al., 1989; Lee et al., 1995). According to data from complete CR sequences, these domains are the most rapidly evolving portions of the mtDNA molecule, with a substitution rate five- to 10-fold higher than the rest of the mtDNA molecule (Aquadro & Greenberg, 1983; Hoelzel et al., 1991; Lopez et al., 1997). The general structure, phylogeny and evolution of the mitochondrial CR of fishes has been extensively studied in many species (Meyer et al., 1990; Lee et al., 1995; Nesbø et al., 1998; Rocha-Olivares et al., 1999; Noell et al., 2001; Murgra et al., 2002; Guo et al., 2003). However, such work in flatfish is still limited (Lee et al., 1995; Tinti et al., 1999; Borsa & Quignard, 2001), especially for those species in the Yellow Sea (China).

In the present study, we report the general structure of the 5'-end sequence of the mtDNA CR in six Pleuronectidae species from the Yellow Sea and one from the North Sea (Belgium). They are the pointhead plaice (*Cleisthenes herzensteini*), stone flounder (*Kareius bicoloratus*), variegated flounder (*Verasper variegatus*), slime flounder (*Microstomus achne*), finespotted flounder (*Pleuronichthys cornutus*), yellow striped flounder (*Pseudopleuronectes herzensteini*) and common dab (*Limanda limanda*). At the same time, comparisons were made between these seven species with 10 other flatfishes: European flounder (*Platichthys flesus flesus*), Adriatic flounder (*Platichthys flesus italicus*), yellowtail flounder (*Limanda ferruginea*), winter flounder (*Pseudopleuronectes americanus*), grey sole (*Glyptocephalus cynoglossus*), American plaice (*Hipploglossoides platessoides*), Japanese flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*), common sole (*Solea vulgaris*) and Klein's sole (*Solea kleini*). Phylogenetic analysis was conducted on these 17 species to evaluate the informative value and usefulness of the 5'-end CR sequence as a molecular marker for flatfish phylogeny and systematics.

## MATERIALS AND METHODS

***Sample collection and DNA extraction.*** – Specimens of *C. herzensteini*, *K. bicoloratus*, *M. achne*, *Ple. cornutus*, *Pse. herzensteini* and *V. variegatus* were collected from local fish markets in Qingdao, China, which were all from the Yellow Sea waters. *Limanda limanda* specimens originating from the North Sea (Belgium) were obtained from a World Fisheries Exhibition in Qingdao, China. All samples were kept at -70ºC before use. Three or four specimens of each species were analyzed in this study. Total genomic DNA was extracted from ~ 0.3 g of muscle tissue using a standard DNA extraction protocol (Sambrook et al., 1989).

***PCR amplification and sequencing.*** – The CR was amplified with a primer set of L15926 (5'-TCAAAGCTTACACCAGTCTTGTAAACC-3') and H16495 (5'-CCTGAAGTAGGAACCAGATG-3') (Meyer et al., 1990). Polymerase Chain Reaction (PCR) was performed in 25 μL volumes which contained 2.5 μL of 10× PCR buffer, 2.0 μL of $MgCl_2$ (2.0 mM), 2.0 μL of each dNTP (2.5 mM), 1.0 μL of each primer (5.0 μM) and 1.0 μL of the genomic DNA template (~50 ng), 1 unit of *Taq* plus polymerase (Sangon, China) and 9.5 μL pure water. PCR amplifications were carried out in a thermal cycler (Eppendorf AG, Germany). The PCR cycling profile was: denaturing at 94ºC for 3 minutes, 33 cycles of denaturing at 94ºC for 50 seconds, annealing at 48ºC for 50 seconds and extending at 72ºC for 1 minute and a final extension at 72ºC for 5 minutes. The double-stranded PCR products were purified and cycle-sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., USA). Electrophoresis was done on an ABI 377XL DNA sequencer. The corresponding sequences (and accession numbers) of *Pla. fle. flesus* (Y14730), *Pla. fle. italicus* (AF034261), *L. ferruginea* (U12064), *Pse. americanus* (U12068), *G. cynoglossus* (U12070), *H. platessoides* (U12059), *Par. olivaceus* (AB028664), *Sco. maximus* (AB069758), *Sol. vulgaris* (AF034262) and *Sol. kleini* (AF034263) were retrieved from GenBank.

***Sequence analysis.*** – The partial CR sequences obtained in this study were aligned with ClustalX 1.81 (Thompson et al., 1997) and then verified manually to minimize mismatches and other errors. Putative tRNA gene regions and conserved sequence blocks or feature segments were localized and verified through alignment with corresponding sequences of other fish species (Lee et al., 1995; Zardoya & Meyer, 1996; Nesbø et al., 1998; Guo et al., 2003). The Extended Terminal Associated Sequence (ETAS) was described and analyzed in comparison with lungfish (Zardoya & Meyer, 1996), percid fish (Nesbø et al., 1998), milkfish (Ravago et al., 2002), carps and zebrafish (Guo et al., 2003). The potential secondary structure of ETAS was analyzed and an estimation of minimum free energies was made (Zuker, 1989) using


Fig. 1. Schematic diagram of structure of the 5'-end mitochondrial DNA (mtDNA) control region (CR) of flatfishes. The tRNA genes, conserved sequence blocks (CSBs) are indicated by blank boxes and the TAS-cTAS motif is indicated by black box. The positions of primers are indicated by arrows.

GeneQuest in DNAStar (Madison, WI USA). Genetic distances were calculated according to the method of Tamura & Nei (1993). Phylogenetic analysis was performed with neighbor-joining (NJ) and maximum parsimony (MP) method using Molecular Evolutionary Genetics Analysis program (MEGA) version 3.0 (Kumar et al., 2004) and the robustness of trees was evaluated by bootstrap analysis with 1,000 replications.

## RESULTS

***General structure of 5'-end control region.* –** PCR amplification and sequencing of 5'-end of CR in the seven flatfish species yielded sequences of 473 to 496 base pairs (bp). Nucleotide sequences were deposited in GenBank under the following accession numbers: *C. herzensteini* (AY671915), *K. bicoloratus* (AY671935), *L. limanda* (AY671920), *M. achne* (AY671925), *Ple. cornutus* (AY671928), *Pse. herzensteini* (AY671933) and *V. variegatus* (AY671918). Figure 1 depicts the general structure of the 5'-end CR for the species studied. An alignment of the representative sequences of the seven species and other 10 species is presented in Figure 2, leading to a 507 bp alignment length. Since the first ~100 bp at the 5'-end belong to the partial tRNA-Thr and complete tRNA-Pro genes, the actual length of 5'-end CR sequence of the seven species was 372 - 395 bp.

The 5'-end of the CR sequences of these species was an AT-rich segment and showed a similar base composition bias (A > T > C > G), which has been observed in some other fishes (Rocha-Olivares et al., 1999; Ravago et al., 2002; Guo et al., 2003), mammals (Sbisá et al., 1997) and insects (Lessinger et al., 2000). The fragment sequenced contained the 5'-end highly-variable domain and a part of the central conserved sequence portion. The sequences consisted of segments with different rates of nucleotide substitution which were similar to the descriptions by Lee et al. (1995) and Tinti et al. (1999). As shown in Figure 2, the two most variable segments (103 - 140 bp) were those adjacent to the tRNA-Pro gene and the larger segment located in the middle of the sequence (185 - 400 bp). Between these two highly-variable segments, there existed a smaller and less variable block of 141 - 184 bp, which contained the ETAS domain. The more conserved segment was at the 3'-end of the sequence (405 - 507 bp), a part of central conserved region (CCR) of the CR. No repetitive unit at the beginning part of this sequence was found in the seven species sequenced. This was similar for the other 10 species, except for *Pla. fle. flesus* and *Pla. fle. italicus,* which both have four 19 bp repeats (Tinti et al., 1999) (Fig. 2). Therefore, no length variation arising from repetitive elements was detected in these species either.

***Extended Terminal Associated Sequence (ETAS) domain.* –** The terminal associated sequence (TAS) motif, TACAT, is presumed to function as a sequence-specific signal for the termination of D-loop strand synthesis (Doda et al., 1981). It has been reported in lungfish (Zardoya & Meyer, 1996), percid fishes (Nesbø et al., 1998), milkfish (Ravago et al., 2002), carps and zebrafish (Guo et al., 2003). In the present study, a TAS motif was found in *C. herzensteini*, *K. bicoloratus, M. achne*, *Pse. herzensteini* and the other 10 species compared (Fig. 2). At the same time, a complementary TAS (cTAS) motif ATGTA was detected just two bp downstream of the TAS motif in all species in this study, except *L. ferruginea*. TAS and cTAS motifs compose a TACAT-ATGTA motif (TAS-cTAS motif). Additionally, an 11 bp palindromic motif - ATGTACATGTA - was also observed in *C. herzensteini, G. cynoglossus*, *K. bicoloratus, M. achne*, *Pla. fle. flesus* and *Pla. fle. italicus* at the TAS-cTAS motif area. Finally, a conserved sequence block overlapping with the TAS-cTAS modif was observed, with the following sequence: (G/A)TATGTAATAACACCATATAT. It was similar to the conserved motif in the 5'-end of the mtDNA CR (CM5'd) in lungfish and percid fish (Zardoya & Meyer, 1996; Nesbø et al., 1998). Through comparisons with the corresponding sequences of other fishes and mammals (Zardoya & Meyer, 1999; Guo et al., 2003; Sbisá et al., 1997), an ETAS of Pleuronectiformes was identified as follows: TACATATATGTAATAACACCAT-TATAGTAACCA- (consisting primarily of the TAS-cTAS motif and CM5'd). The ETAS has about 94% similarity to that in Guo et al. (2003) (Table 1). A potential stable hairpin-loop structure in the ETAS is predicted based on the minimum free energies of folding (Fig. 3), which may contribute to the TAS function.

***Central conserved sequence block (CSB) domains.* –** Lee et al. (1995) reported a CSB-D of the CCR in some teleost fish. Recently, however, Guo et al. (2003) identified three CSBs: CSB-D, CSB-E and CSB-F in the complete CR sequence from carps and zebrafish. Based on comparisons with the previous studies (Lee et al., 1995; Zardoya & Meyer, 1996; Guo et al., 2003), we located the partial CSB-D at the 3'-end of the sequence and CSB-F, which was identified as follows: ATGTAGTAAGAGCCTACCAACC (Fig. 2). It showed high similarities to CSB-F that was described by Guo et al. (2003). In addition, the "GTGGG" box that is commonly found in most teleost fish (Samonte et al., 2000; Ravago et al., 2002) was also identified at the 3'-end.

***Phylogenetic analysis.* –** The Tamura-Nei (1993) distances which were based on 5'-end CR sequences (excluding the tRNA part) among all the species are presented in Table 2. The average distances among these four families range from 0.399 to 0.580, with the smallest value occurring between the families Pleuronectidae and Paralichthyidae. While intra-generic distances between *Pse. herzensteini* and *Pse. americanus* and between *Pla. fle. flesus* and *Pla. fle. italicus* were very small, the intra-generic distance between *L. limanda* and *L. ferruginea* and *Sol. vulgaris* and *Sol. kleini* were very large. Their intra-generic distances were even larger than some of inter-generic ones. Consequently, while the species in the genera *Platichthys* and *Pseudopleuronectes* clustered together in the neighbor-joining (NJ) tree, *L. limanda* and *L. ferruginea* grouped separately on different branches, indicating significant divergence between them at molecular level (Fig. 4). Notably, *Ple. cornutus* showed significant large distances to all other species in the

Table 1. Comparison of the Extended Terminal Associated Sequence (ETAS), conserved motif (CM5'd) and complete conserved sequence blocks (CSB-F) in some teleost fishes. Dots (.) indicate nucleotides identical to the first line.

| | Fish | Sequence Comparison | Source |
|---|---|---|---|
| ETAS | Flatfish | TACATATATGTAATAACACCATTATAGTAACCA | Present study |
| | Carps | .....T..T..... | Guo et al. (2003) |
| CM5'd | Flatfish | GTATGTAATAACACCATATA | Present study |
| | Eel | A.....A..TTA.....C. | Inoue et al. (2001) |
| | Lungfish | C.....T.TCGTA.....TA. | Zardoya & Meyer (1996) |
| | Perch | .....T.T.....C. | Nesbø et al. (1998) |
| | Puffer fish | A.....T..T.C.....TC. | Elmerot et al.(2002) |
| | Salmon | C.....TT.C..... | Hurst et al. (1999) |
| CSB-F | Flatfish | ATGTAGTAAGAGCCTACCAACC | Present study |
| | Carps | .....—A.C-..... | Guo et al. (2003) |
| | Perch | .....-.T. | Nesbø et al. (1998) |
| | Salmon | .....G.....G | Hurst et al. (1999) |
| | Sardine | .....A.....A.....G.....G | Inoue et al. (2000) |
| | Zebrafish | ....CA.....G.....—A.C-..... | Guo et al. (2003) |

Pleuronectidae (its distance to all other species was 0.377 on average, while the average distance among all the species in the family was 0.209), indicating its significant divergence from the other species in this family, as supported by its bootstrap value. Although differing branch topologies and divergences of *Ple. cornutus* from the other species in this family were observed, all 10 genera belonging to the Family Pleuronectidae always clustered in a monophyletic group on both the NJ and MP trees generated. Meanwhile, the families Paralichthyidae, Scophthalmidae and Soleidae grouped separately in both the trees (Figs. 4 & 5).

## DISCUSSION

The 5'-end CR sequences of the fish determined in this study as well as the other 10 species previously sequenced species clearly show that they generally share the same architecture of the 5'-end half of CR in other fish (Lee et al., 1995): variable 5'-end portion and highly-conserved 3'-end (CCR). It is a common phenomenon for segments directly adjacent to tRNA genes to show the highest rates of variation (Saccone et al., 1991; Tinti et al., 1999). This high level of variation is usually due to the high rate of nucleotide substitutions and the existence of repetitive elements. Length variation of the CR observed in many species usually results from the presence of varying numbers of tandem repeats. They exist either at the 5'-end of the CR, as seen in perch, milkfish, European flounder, Adriatic flounder, white sturgeon and Atlantic cod (Lee et al., 1995; Nesbø et al., 1998; Tinti et al., 1999; Saitoh et al., 2000; Ravago et al., 2002), or at the 3'-end of the CR, as seen in some other flounders such as the Japanese, winter and yellowtail flounders (Lee et al., 1995). While tandem repeats were not found at the 5'-end, their presence or absence at 3'-end of the CRs of the seven species studied here would be revealed when the complete sequences of their CR are available.

Guo et al. (2003) reported that the ETAS of carps and other fish as: TACATAT-ATGTATTATCACCAT-TATATTAACCA and pointed out that the TAS-cTAS motif (TACAT-ATGTA) could form a stable hairpin-loop and may be the main body of the conserved ETAS. In this study, the similar ETAS of the Pleuronectiformes were identified as: TACATATATGTAATAACACCAT-TATAGTAACCA. The structure of the two ETAS were predicted and compared. Both motifs form potential stem-loop structures and the most conserved parts were the bases that were predicted to form the stem (Fig. 3). The estimate of minimum free energies of ETAS established in our study (-2.12 kJ/M) was lower than that (- 0.93 kJ/M) in Guo et al. (2003). This implies that the potential hairpin-loop structure of the Pleuronectiformes might be more stable. The core sequence of the ETAS may be ATATAT-ATATAT. Additionally, Nesbø et al. (1998) identified a conserved motif at the 5'-end CR of percid fish (CM5'd), which was similar to that of lungfish and other vertebrates (Zardoya & Meyer, 1996). In this study, a conserved motif in flatfish which is similar to the CM5'd of these and other species (Hurst et al., 1999; Inoue et al., 2001; Elmerot et al., 2002) was identified (Table 1).

While the CSB-B, CSB-C, CSB-D, CSB-E and CSB-F blocks of the CCR were identified in some mammals (Southern et al., 1988) and birds (Randi & Lucchini, 1998; Saunders & Edwards 2000), only CSB-D has been identified in some teleost fishes (Lee et al., 1995). However, Guo et al. (2003) recently identified blocks of CSB-D, CSB-E and CSB-F from six carps and zebrafish. CSB-E and CSB-F blocks were located between a big variable block and the CSB-D in the CCR in cyprinid species. In our current study, the partial CSB-D sequence (TTCCTGG) was found at the 3'-end of the sequence, which was identical to the first half of the CSB-D in cichlid, perch and freshwater sardines (Lee. et al., 1995; Nesbø et al., 1998; Samonte et al., 2000). In comparison with other fish including cyprinids, perch, zebrafish, sardine, salmon (Hurst et al., 1999; Inoue et al., 2001), we found the CSB-F sequence in this segment across almost all these Pleuronectidae species (Fig 2). And its sequence (ATGTAG-TAAGAG-CCTACCAACC) was very similar to the CSB-F in carps and zebrafish (ATGTAGTAAGAGAC-CACC) as indicated by Guo et al. (2003), but with some variation. Both of them have the unique key sequence of ATGTAGGAGACCACC.

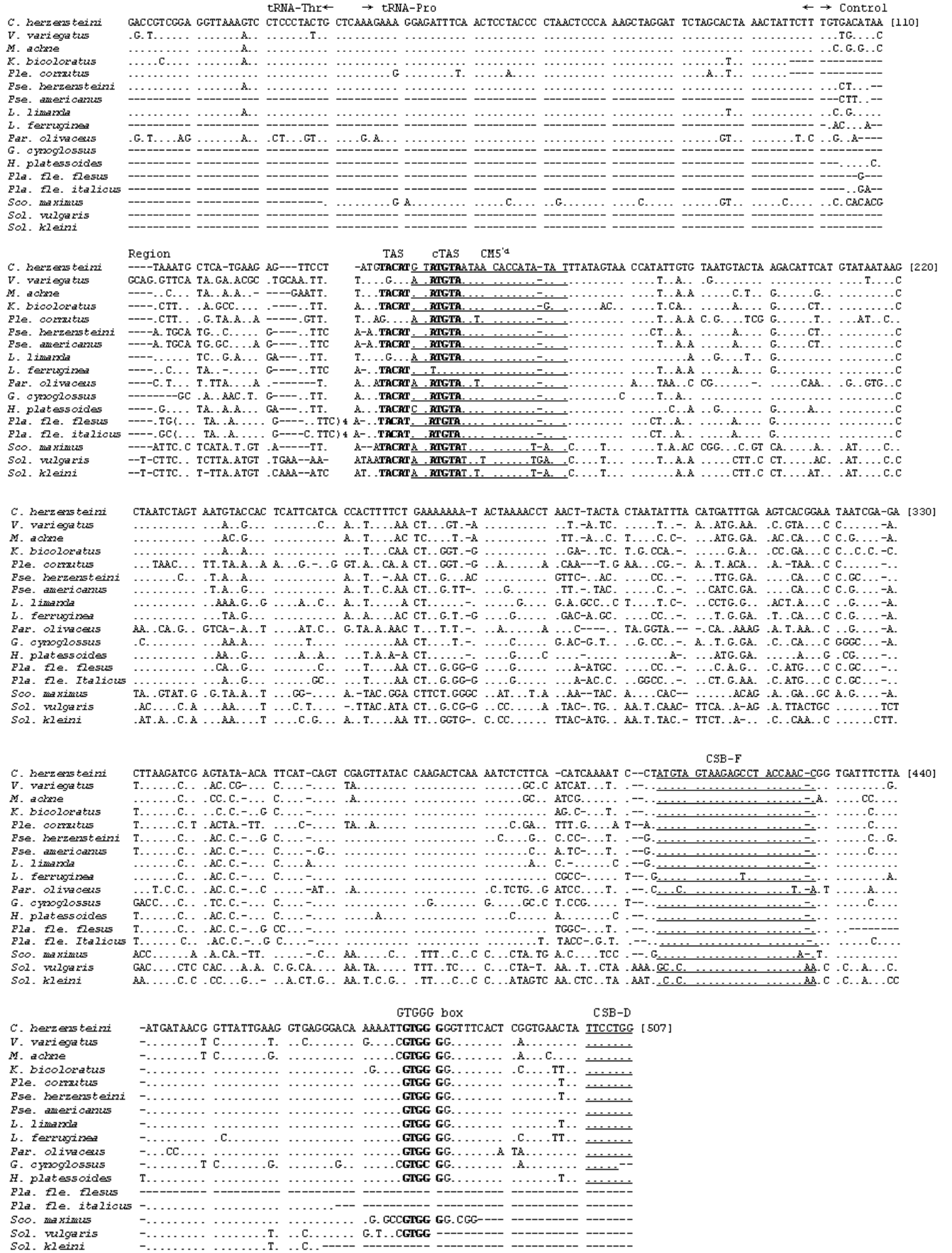


Fig. 2. Sequence alignment of the 5'-end mitochondrial control region (CR) of 17 flatfish species. Dots indicate a nucleotide identity to the sequence on the top and dashes indicate introduced gaps by alignment. Boundaries between putative genes/regions are indicated by arrows. Sequences corresponding to TAS, cTAS and GTGGG box are boldfaced. CM5'd, CSB-F and CSB-D are underlined. Tandem repeated arrays are parenthesized. The GenBank accession numbers of *C. herzensteini*, *K. bicoloratus*, *L. limanda*, *M. achne*, *Ple. cornutus*, *Pse. herzensteini* and *V. variegatus* are AY671915, AY671935, AY671920, AY671925, AY671928, AY671933 and AY671918 respectively.

Table 2. The distance matrix of the aligned 5'-end sequences of control region (CR) among 17 species from the Order Pleuronectiformes.

| Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Cleisthenes herzensteini* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Verasper variegatus* | 0.251 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Microstomus achne* | 0.218 | 0.166 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Kareius bicoloratus* | 0.203 | 0.221 | 0.219 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Pleuronichthys cornutus* | 0.333 | 0.331 | 0.344 | 0.313 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Pseudopleuronectes herzensteini* | 0.214 | 0.218 | 0.205 | 0.158 | 0.340 | - | - | - | - | - | - | - | - | - | - | - | - |
| *Pseudopleuronectes americanus* | 0.218 | 0.201 | 0.178 | 0.179 | 0.320 | 0.078 | - | - | - | - | - | - | - | - | - | - | - |
| *Limanda limanda* | 0.172 | 0.235 | 0.173 | 0.249 | 0.309 | 0.210 | 0.202 | - | - | - | - | - | - | - | - | - | - |
| *Limanda ferruginea* | 0.197 | 0.230 | 0.166 | 0.170 | 0.319 | 0.142 | 0.160 | 0.190 | - | - | - | - | - | - | - | - | - |
| *Glyptocephalus cynoglossus* | 0.228 | 0.210 | 0.200 | 0.220 | 0.375 | 0.199 | 0.224 | 0.252 | 0.181 | - | - | - | - | - | - | - | - |
| *Hippoglossoides platessoides* | 0.143 | 0.236 | 0.204 | 0.211 | 0.329 | 0.194 | 0.190 | 0.122 | 0.179 | 0.235 | - | - | - | - | - | - | - |
| *Platichthys flesus flesus* | 0.234 | 0.243 | 0.219 | 0.170 | 0.434 | 0.165 | 0.195 | 0.248 | 0.136 | 0.225 | 0.207 | - | - | - | - | - | - |
| *Platichthys flesus italicus* | 0.233 | 0.238 | 0.217 | 0.162 | 0.397 | 0.163 | 0.199 | 0.247 | 0.142 | 0.210 | 0.201 | 0.067 | - | - | - | - | - |
| *Paralichthys olivaceus* | 0.362 | 0.389 | 0.373 | 0.405 | 0.415 | 0.420 | 0.386 | 0.375 | 0.386 | 0.418 | 0.352 | 0.482 | 0.426 | - | - | - | - |
| *Scophthalmus maximus* | 0.547 | 0.585 | 0.548 | 0.554 | 0.526 | 0.583 | 0.552 | 0.537 | 0.544 | 0.570 | 0.523 | 0.655 | 0.576 | 0.529 | - | - | - |
| *Solea vulgaris* | 0.621 | 0.548 | 0.566 | 0.611 | 0.587 | 0.587 | 0.543 | 0.572 | 0.581 | 0.616 | 0.570 | 0.655 | 0.581 | 0.587 | 0.447 | - | - |
| *Solea kleini* | 0.559 | 0.523 | 0.514 | 0.547 | 0.580 | 0.529 | 0.490 | 0.538 | 0.527 | 0.574 | 0.575 | 0.587 | 0.529 | 0.573 | 0.517 | 0.247 | - |

The mitochondrial CR is one of the most frequently utilized sequences for both intra- and inter-specific genetic studies in fishes. In phylogenetic studies, the mtDNA CR is considered and has been proven to be useful at low and medium but not at high taxonomic levels due to its fast evolutionary rate, (Chen et al., 1998; Tinti et al., 1999; Samonte et al., 2000). For example, Tinti et al. (1999) reconstructed the phylogenetic relationships of nine flatfish (including families of Pleuronectidae, Bothidae and Soleidae) at the family level using 5'-end CR sequence data and found that the species of these three families were clearly separated into three groups in phylogenetic trees and the resultant phylogenetic relationships were concordant with conventional ones.

Due to the relative small distance between Pleuronectidae and Paralichthyidae species, the two families appear closer and more related in phylogenetic trees than to other species. This result supports their grouping in the suborder of Pleuronectoidei, which was established previously based on morphologic characters (Chapleau, 1993; Nelson, 1994; Li & Wang, 1995). This finding was also consistent with that based on the nucleotide sequence data of the 12S and 16S mitochondrial ribosomal genes (Berendzen & Dimmick, 2002). Since this study analyzed eight more species than Tinti et al.'s (1999), it was not surprising that clear separation of four families (Pleuronectidae, Paralichthyidae, Scophthalmidae and Soleidae) was retrieved in the tree. Our findings supported Tinti et al.'s (1999) opinion and indicate the utility of this sequence in the analysis or reconstruction of phylogenetic relationships for Pleuronectiformes at the family level.

However, within the Family Pleuronectidae, significant divergence was observed between *L. limanda* and *L. ferruginea* at the sequence level. Cooper and Chapleau (1998) indicated that, while species in the genera *Platichthys*, *Pleuronectes* and *Pseudopleuronectes* were all monophyletic within the Family Pleuronectidae, six species in the genus *Limanda* were clearly not monophyletic due to homoplasy observed from morphology. The six species formed three sub-branches in both consensus trees of the 53 Pleuronectidae species and the tree of the Pleuronectidae species. The three sub-branches included: 1) *L. punctatissima* and *L. proboscidea*: 2) *Limanda ferruginea* and *L. aspera* and 3) *Limanda sakhalinensis* and *L. limanda.* Our data was in line with their result in that *L. ferruginea* and *L. Limanda* were not in the same branch.

Additionally, it was remarkable that the distances of *Ple. cornutus* from all other species in Pleuronectidae is relatively large. This phenomenon was also observed in our 16s rRNA and cytochrome *b* (cyt *b*) data of these flatfishes (unpublished data), indicating significant divergence of this species from other species, on both highly variable and conserved sequences. However, according to a cladistic analysis based on morphological and osteological characters done by Cooper and Chapleau (1998), *Ple. cornutus* and other species in genus *Pleuronichthys* do not show that much divergence from other genera.

The discrepancy of phylogenetic relationships of *Ple. cornutus* from all other species in molecular and morphology characteristics may require more studies or reassessment of the systematic value of some morphological characters. Finally, the 10 genera of the Family Pleuronectidae grouped firmly in a big branch in the phylogenetic tree, supporting the fact that they are of monophyletic origin.

In summary, the structural arrangement of the 5'-end CR sequence appeared very similar and some conserved sequence

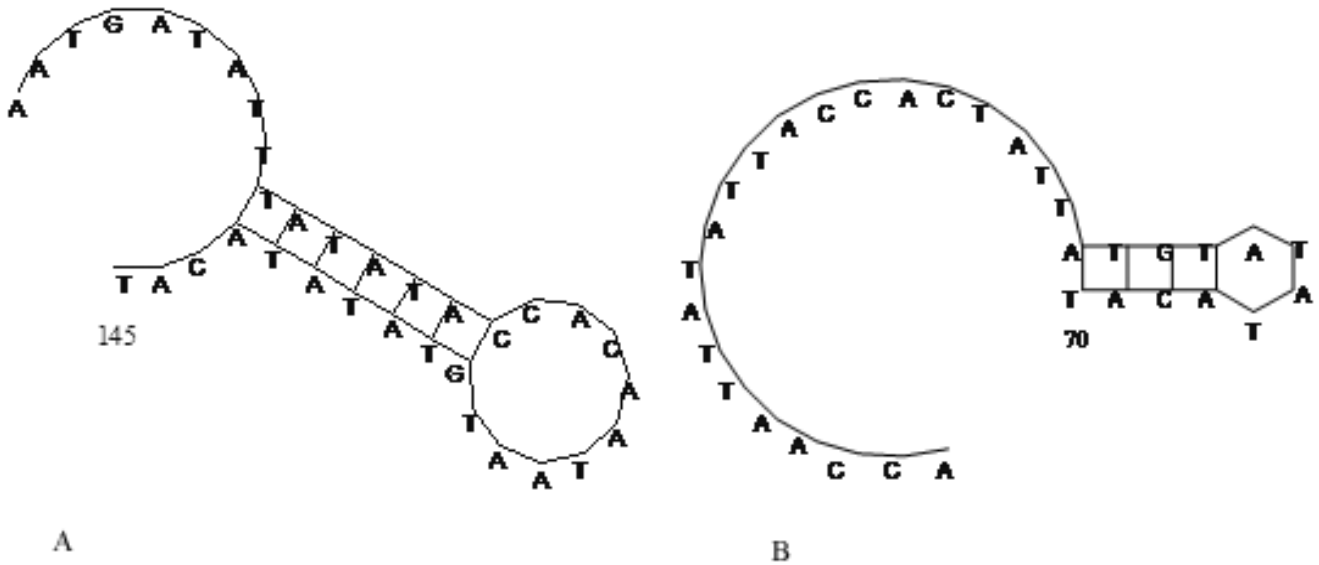


Fig. 3. Potential secondary structures for Extended Terminal Associated Sequence (ETAS), from aligned sequences. A) potential secondary structure of the ETAS (5'-TACATATATGTAATAACACCATATATTTATAGTAA-3') from base pairs 145 through to 180 from the aligned sequences in Fig. 2 (minimum energy: -2.12 KJ/M), B) potential secondary structure of the ETAS (5'-TACATATATGTATTATCACCATTATATTAACCA-3') from base pairs 70 through to 100 in the aligned sequences of Guo et al., 2003 (minimum energy: -0.93 KJ/M)**.** The numbers indicate the starting points in the sequences.

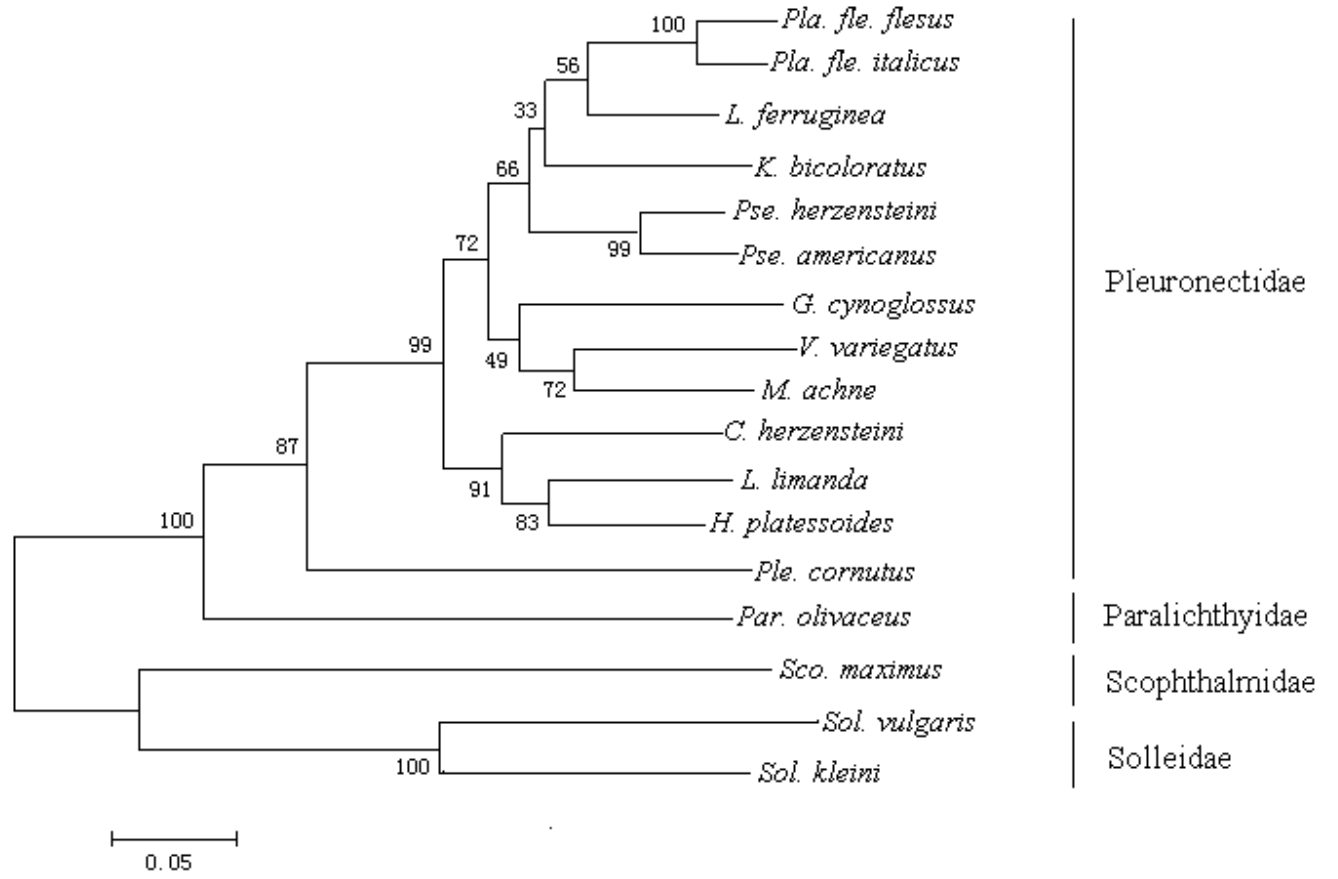


Fig. 4. Bootstrapped neighbor-joining (NJ) tree based on the 5'-end sequences of the mitochondrial control region (CR) of 17 flatfish species. Numbers above branches indicate bootstrap values for 1,000 replicates. Scale bar represents a genetic distance of D = 0.05, calculated according to the method of Tamura & Nei (1993).

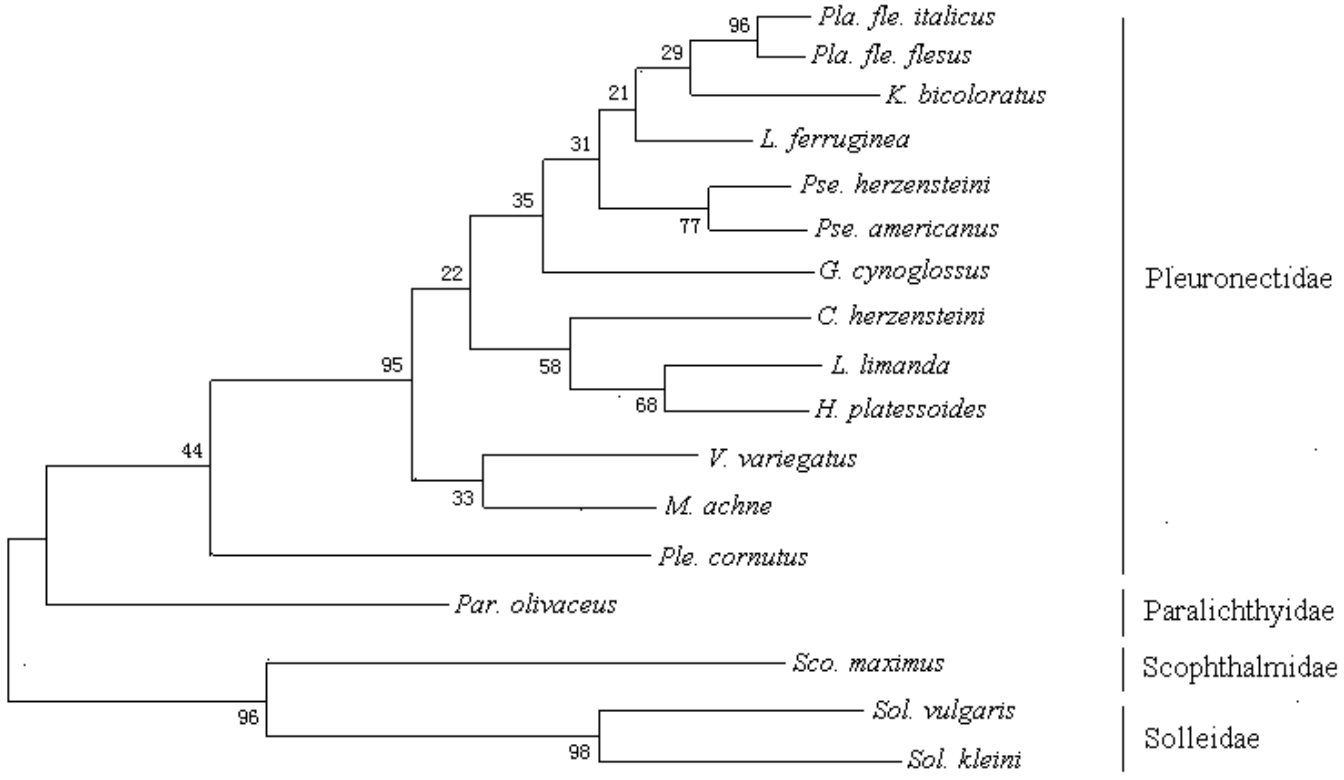


Fig. 5. Maximum parsimony (MP) tree based on the 5'-end sequence of the mitochondrial control region (CR) of 17 flatfish species. Numbers above branches indicate bootstrap values for 1,000 replicates.

blocks were detected across almost all species studied in this paper. The molecular data of this fragment in the species studied here showed four evolutionary lineages which corresponded to the four families in the Order Pleuronectiformes. This study also showed that molecular phylogenetic relationships based on this sequence were highly reliable and hopefully the results will inspire further studies of the analysis and reconstruction of phylogenetic relationships in Pleuronectiformes species along the West Pacific Ocean Coast.

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