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# Mitochondrial genomic investigation of flatfish monophyly

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ABSTRACT

We present the first study to use whole mitochondrial genome sequences to examine phylogenetic affinities of the flatfishes (Pleuronectiformes). Flatfishes have attracted attention in evolutionary biology since the early history of the field because understanding the evolutionary history and patterns of diversification of the group will shed light on the evolution of novel body plans. Because recent molecular studies based primarily on DNA sequences from nuclear loci have yielded conflicting results, it is important to examine phylogenetic signal in different genomes and genome regions. We aligned and analyzed mitochondrial genome sequences from thirty-nine pleuronectiforms including nine that are newly reported here, and sixty-six non-pleuronectiforms (twenty additional clade L taxa [Carangimorpha or Carangimorpharia] and forty-six secondary outgroup taxa). The analyses yield strong support for clade L and weak support for the monophyly of Pleuronectiformes. The suborder Pleuronectoidei receives moderate support, and as with other molecular studies the putatively basal lineage of Pleuronectiformes, the Psettodoidei is frequently not most closely related to other pleuronectiforms. Within the Pleuronectoidei, the basal lineages in the group are poorly resolved, however several flatfish subclades receive consistent support. The affinities of *Lepidoblepharon* and *Citharoides* among pleuronectoids are particularly uncertain with these data.



## 1. Introduction

Flatfishes (Pleuronectiformes) are a distinctive group of vertebrates characterized by bilateral asymmetry (Chapleau, 1993; Frazzetta, 2012). All but three extant species of flatfishes (>700 species, 14 families and 134 genera) are assigned to the suborder Pleuronectoidei (Munroe, 2005; Nelson, 2006). The three known species of *Psettodes* form the pleuronectiform suborder Psettodoidei. The remarkable body plan of flatfishes fed debate questioning the adequacy of natural selection as a theory of anatomical diversification, and much speculation on the speed of such a change in part due to the lack of extant intermediates (Janvier, 2008; Mivart, 1871). Only recently have intermediate flatfish forms been recognized in the fossil record (Friedman, 2008, 2012).

Complicating the topic of flatfish evolutionary origins, support for the monophyly of Pleuronectiformes is not universal nor has it received clear support in phylogenetic studies. Evidence for flatfish paraphyly was offered in several studies (Amaoka, 1969; Chabanaud, 1949; Norman, 1934) predating a cladistic synthesis that concluded in support of the monophyly of the group (Chapleau, 1993). The conclusion of Chapleau (1993) of pleuronectiform monophyly has been widely accepted and in this light, results of molecular-based studies that offer evidence for flatfish paraphyly are intriguing (Betancur-R. et al., 2013a, 2013b; Campbell et al., 2013a; Chen et al., 2003; Dettai and Lecointre, 2005; Li et al., 2009; Near et al., 2012, 2013; Smith and Wheeler, 2006). When molecular evidence does provide support for monophyly of the flatfishes, the result is often sensitive to the particular combination of analyses and datasets examined (Betancur-R. et al., 2013b; Campbell et al., 2014). The debate surrounding what DNA sequences say about monophyly of flatfishes continues (Betancur-R. and Ortí, 2014; Campbell et al., 2014). While GC-biased base composition can be shown to play a role in disrupting pleuronectiform monophyly when particular taxa are examined, that effect does not explain the

*Abbreviations:* 1, first codon positions of aligned proteins; 2, second codon positions of aligned proteins; 3, third codon positions of aligned proteins; DDBJ, DNA Data Bank of Japan; DNA, deoxyribonucleic acid; EMBL, European Molecular Biology Laboratory; Γ, four-category gamma distributed rate variation among sites; GTR, general time reversible model of nucleotide evolution; ML, maximum likelihood; MSA, multiple sequence alignment; n, nucleotide; ND6, NADH-ubiquinone oxidoreductase chain 6; PCR, polymerase chain reaction; R, ribosomal RNA; RNA, ribonucleic acid; rRNA, ribosomal RNA; RY, purine and pyrimidine recoding; T, transfer RNA; tRNA, transfer RNA.

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consistent placement of the genus *Psettodes* (spiny turbots) outside a restricted pleuronectiform clade (Campbell et al., 2013a). The placement of *Psettodes* apart from other pleuronectiforms may be the product of incomplete lineage sorting and/or the inability to correctly infer gene trees in nuclear datasets focusing on pleuronectiform monophyly (Campbell et al., 2014).

A critical review of the three putative pleuronectiform synapomorphies identified by Chapleau (1993) shows that those traits are not shared by *Psettodes* (Chabanaud, 1937; Nelson, 2006). The only morphological characters uniting Pleuronectiformes appear to be the correlates of bilateral asymmetry, which take a distinct form in *Psettodes* (Friedman, 2008). To date, phylogenetic studies show that the monophyly of pleuronectoids is well supported (Campbell et al., 2013a) and that the evolutionary affinities of all flatfishes (Psettodoidei and Pleuronectoidei) are with the Carangimorpha or clade L *sensu* Chen et al. (2003). Molecular evidence highlighted a close relationship between carangids and pleuronectids first with whole mitochondrial genome (mitogenome) data (Miya et al., 2003). This placement is well established and consistently supported (Betancur-R. et al., 2013a; Chen et al., 2003, 2007; Little et al., 2010; Miya et al., 2003; Near et al., 2012; Smith and Craig, 2007; Smith and Wheeler, 2006; Wainwright et al., 2012). Clade L currently contains flatfishes plus an array of perciform taxa with diverse morphologies including Toxotidae (archerfishes), Carangidae (jacks), Centropomidae + Latidae (snooks, Nile perches and allies), Xiphiidae (swordfish), Istophoridae (billfishes), Polynemidae (threadfins), Echeneidae (remoras), Coryphaenidae (dolphin fishes), Rachycentridae (cobia), Sphyraenidae (barracudas), Menidae (moonfish), and *Lactarius* (false trevally) (Campbell et al., 2013a).

Flatfishes then are in a curious position. A monophyletic 'clade L' is consistently found with high indices of support in molecular studies, although it contains a diverse array of morphological forms. In contrast, a monophyletic Pleuronectiformes receives only weak and inconsistent support in some concatenated phylogenetic analyses (Betancur-R. et al., 2013b) and a single gene tree to species tree analysis (Betancur-R. and Ortí, 2014) despite the striking bilateral asymmetry characteristic of all species in the group. In addition, evaluation of different species trees from gene tree frameworks, datasets without missing data, accommodating for divergent base composition, and different configurations of concatenated analyses of nuclear gene data yield paraphyletic arrangements of the two main pleuronectiform lineages (Betancur-R. et al., 2013a, 2013b; Campbell et al., 2013a, 2014).

Here we report results of a thorough examination of phylogenetic signal in mitochondrial genomes to infer pleuronectiform inter- and intra-relationships. Mitogenomes have a long history of use in fish molecular phylogenetics and have been proven effective in resolving many areas of the fish tree of life (e.g. Campbell et al., 2013b; Doosey et al., 2009; Inoue et al., 2001, 2003; Miya and Nishida, 2000; Miya et al., 2003, 2010, 2013; Poulsen et al., 2013; Saitoh et al., 2003) while offering a number of practical advantages for phylogenetic inference (e.g. extremely conserved organization, uniparental/haploid inheritance, and large number of characters and variable sites inherited as a single, non-recombining unit). Because mitochondrial sequences show faster rates of substitution and smaller effective population size when compared to nuclear genomes, they have the potential to retain phylogenetic signal for diversification events that nuclear sequences may not (Charlesworth, 2009; Felsenstein, 2004). Our central goal is to establish to what extent patterns of mitogenomic variability among living flatfishes and their close relatives are congruent or in contradiction with expectations derived from flatfish monophyly.

## 2. Materials and methods

Mitogenomes from twenty non-pleuronectiform clade L taxa selected to maximize the diversity of sampled lineages (Miya et al., 2013) were obtained from GenBank (Supplemental Table S1). An additional forty-six candidate outgroups following Campbell et al. (2013a) were obtained from available mitogenome sequences. Among pleuronectiforms, we included all mitogenomic sequences available in GenBank removing a duplicate mitogenome sequence.We then targeted maximal divergences in unrepresented lineages to increase the accuracy of phylogenetic inference (Hillis, 1998; Hillis et al., 2003; Pollock et al., 2002). Mitogenome sequencing was conducted through long PCR then Sanger sequencing of short amplicons (Miya and Nishida, 1999). Multiple sequence alignments (MSAs) were made for the protein-coding genes excluding ND6 due to compositional heterogeneity. First, amino acid sequences were aligned with MUSCLE version 3.8.31 (Edgar, 2004a, 2004b) and the corresponding DNA sequences aligned following the amino acid alignment. Ribosomal RNA (rRNA) sequences were aligned to an existing alignment (Miya et al., 2013) and a new and transfer RNA (tRNA) alignment was made with MUSCLE version 3.8.31 and regions of uncertain positional homology in alignments were excluded from subsequent analyses. To determine if saturation exists in our alignments we carried out a test of saturation (Xia and Lemey, 2009; Xia et al., 2003) with DAMBE version 5.3.109 (Xia, 2013).

We then conducted maximum likelihood (ML) phylogenetic analyses using RAxML version 8.0.0 under GTR+Γ model of nucleotide evolution with automatic stopping of bootstrap replicates (Stamatakis and Ott, 2008) using twenty-three different configurations. These alternative configurations differ in sequence region inclusion/exclusion, coding of third codon positions as purines and pyrimidines ($1_N2_N3_{RY}$) to improve phylogenetic performance in the case of saturation and compositional bias (Chen and Mayden, 2009; Phillips and Penny, 2003; Phillips et al., 2004; Saitoh et al., 2006) exclusion of third codon positions ($1_N2_N$), and partitioning scheme. The full dataset was partitioned by codon positions for each gene with third codon position sites included, recoded, or removed, rRNA (R), and tRNA (T) partitions (noted as: $1_N2_N3_N$RT, $1_N2_N3_{RY}$RT, and $1_N2_N$RT). In addition, we used partition schemes identified with PartitionFinder (Lanfear et al., 2012) on eight alternative analysis schemes and conducted ML phylogenetic analyses on the un-partitioned datasets. Support from each component of the dataset was investigated separately such as protein coding genes by codon positions only, rRNA only, and rRNA + tRNA. To objectively choose the partitioning that best fits observed sequence variation, we used the Bayesian information criterion (BIC) (Schwarz, 1978) as it was the default selection criterion used by PartitionFinder. For twenty analysis configurations we computed BIC for those datasets which contained protein coding gene data or protein coding gene data and rRNA and tRNA data. For the full alignments ($1_N2_N3_N$RT, $1_N2_N3_{RY}$RT, and $1_N2_N$RT) that were partitioned by codon position and rRNA and tRNA, we conducted Shimodaira–Hasegawa (SH) tests (Shimodaira and Hasegawa, 1999) in RAxML version 8.0.19 using the same specifications as the previous analyses (Stamatakis and Ott, 2008) to evaluate whether or not the alternative phylogenetic hypotheses are significantly different. A constrained topology of a monophyletic Centropomidae + Pleuronectoidei as found by Campbell et al. (2013a) was tested against the best tree generated by unconstrained analyses while allowing model parameters to be estimated separately for each tree.

## 3. Results

A total of nine new mitogenome sequences from flatfishes were determined for this study and accessioned in the DDBJ/GenBank/EMBD under accessions AP014586–AP014594. Details of gene composition and organization, and molecular evolution of these newly available mitogenomes will be presented elsewhere.

Our alignment consists of 105 total taxa. Our total alignment of unrecoded data ($1_N2_N3_N$RT) contains 13,742 sites with 9091 distinct alignment patterns. The proportion of missing data was 0.21%. Tests of saturation indicated that third codon positions were saturated, but not other codon position partitions, rRNA or tRNA (Supplementary Table 2). Partitioned ML analyses of the complete dataset partitioned