Gene 551 (2014) 176–182

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Mitochondrial genomic investigation of flatfish monophyly

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ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form 11 July 2014
Accepted 26 August 2014
Available online 27 August 2014

Keywords:
Carangimorpharia
Pleuronectiformes
Mitogenomes
Phylogeny
Partitioned analysis
Incomplete lineage sorting

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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http://dx.doi.org/10.1016/j.gene.2014.08.053
0378-1119/

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_NRT). 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_NRT) 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

by: codon positions for protein coding genes (with and without recoding of third codons), ribosomal RNAs, and transfer RNAs ($1_N2_N3_N$RT and $1_N2_N3_{RY}$RT) yield a monophyletic Pleuronectiformes (Fig. 1; Table 1) with low support (bootstrap values of 8% and 22%, respectively), monophyletic Pleuronectoidei with low support (20% and 46%, respectively) and a monophyletic clade L with high support (100% in both cases). Exclusion of the substitution saturated third codon positions (1_N2_NRT) did not result in a monophyletic Pleuronectiformes or Pleuronectoidei, but had high support for clade L (100%).

Pleuronectiform monophyly is evident in only eight of the twenty-three analysis configurations (Table 1) with all those cases showing invariably low support for monophyly of the group (bootstrap support < 23%, average of 12%). Support for Pleuronectoidei is common, found in eighteen of twenty-three analyses, but weak (bootstrap support < 46%). Support for clade L is found in twenty-two of the twenty-three analyses, and bootstrap support for clade L is frequently greater than 97%. A monophyletic clade L was not found only with a single partition analysis of tRNA.

Considering only the full datasets ($1_N2_N3_N$RT, $1_N2_N3_{RY}$RT, and 1_N2_NRT) partitioned by codon position and RNA type and relationships within Pleuronectiformes, we find evidence of Paralichthyidae comprising two distinct lineages. Otherwise family level divisions within Pleuronectoidei were monophyletic. Strong support from the full datasets partitioned by codon position and RNA type ($1_N2_N3_N$RT, $1_N2_N3_{RY}$RT, and 1_N2_NRT) indicates that the genus *Paraplagusia* is nested with *Cynoglossus*. Pleuronectoidei in our analyses is comprised of several stable groupings which are uncertain in affinity at higher levels. Pleuronectidae is highly supported and most closely related to Paralichthyidae (*Paralichthys* + *Pseudorhombus*). Bothidae is highly

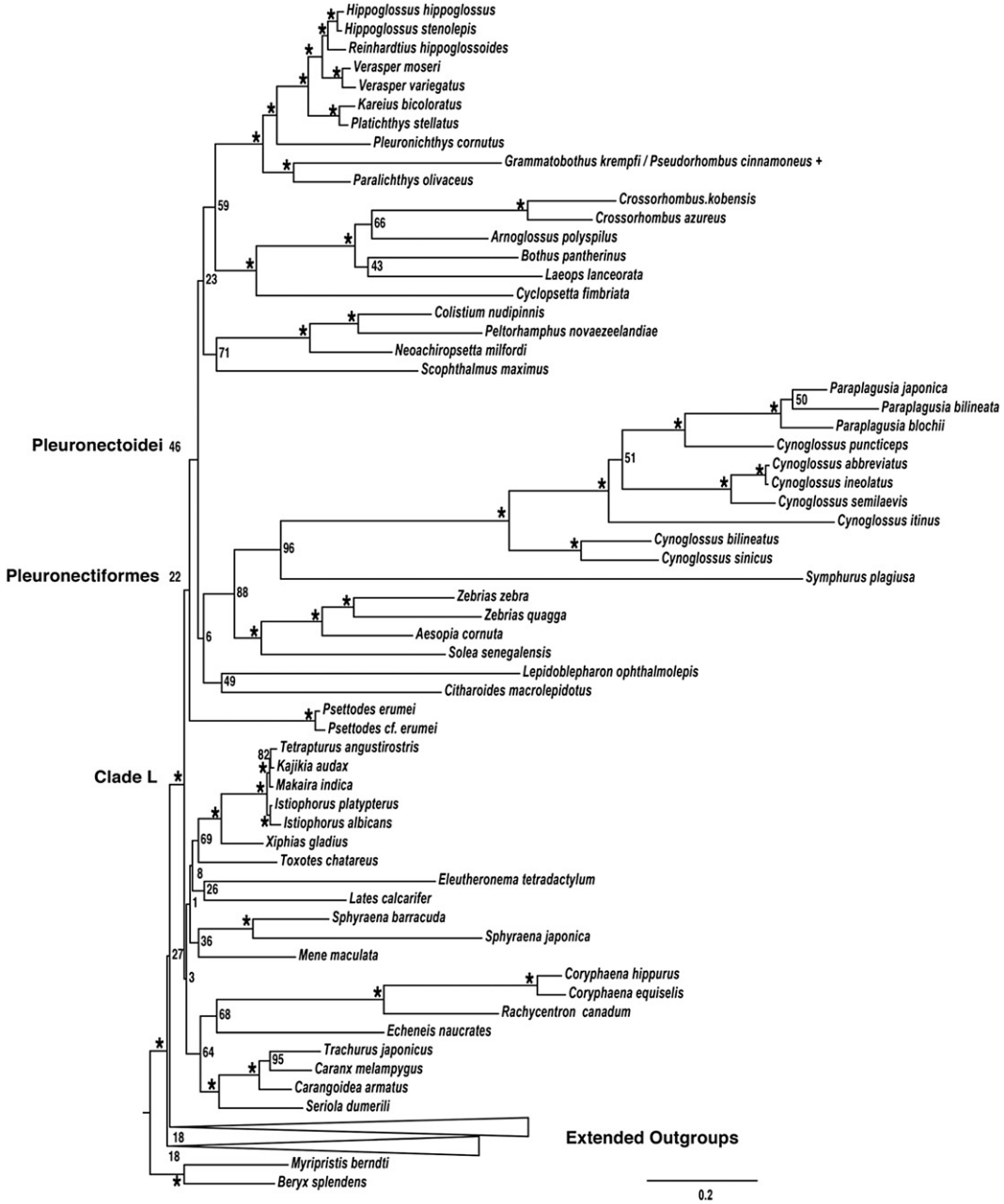

Fig. 1. A maximum likelihood (ML) tree generated in RAxML version 8.0.0 under a GTR+Γ model of nucleotide evolution. Mitogenomes were partitioned by codon position with third codons recoded, rRNA, and tRNA ($1_N2_N3_{RY}$RT). Values at nodes indicate bootstrap support values, and asterisk (*) indicates a value of 100%. +Sequences for *Grammatobothus krempfi* and *Pseudorhombus cinnamoneus* retrieved from GenBank were identical and only one copy was included in this study.

Table 1
Summary of the twenty-three analyses conducted for this study. All analyses were conducted on the same alignment under a GTR+Γ model of evolution in RAxML version 8.0.0. Data included, purine/pyrimidine recoding of third codon positions, and partitioning scheme varied between analyses. Partition schemes were single, subjective, or generated in PartitionFinder. Partitions are described with codon positions appended if applicable (e.g. atp61, atp62, and atp63). If the Pleuronectiformes, Pleuronectoidei, or clade L/Carangimorpha were found to be monophyletic, the associated bootstrap support is reported.

Included data	Coding	Included partitions	Partition scheme	Pleuronectiformes		Pleuronectoidei		Clade L/Carangimorpha	
				Monophyletic	Support	Monophyletic	Support	Monophyletic	Support
Protein coding genes	$1_N2_N3_N$	First, second, third codon positions	Single	No	–	Yes	10	Yes	98
Protein coding genes	$1_N2_N3_{RY}$	First, second, third codon positions	Single	No	–	Yes	13	Yes	99
Protein coding genes	1_N2_N	First and second codon positions	Single	Yes	16	Yes	32	Yes	99
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_N$	First, second, third codon positions, rRNA, tRNA	Single	No	–	Yes	12	Yes	99
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_{RY}$	First, second, third codon positions, rRNA, tRNA	Single	No	–	Yes	27	Yes	100
Protein coding genes, 12S, 16S, tRNA	1_N2_N	First and second codon positions, rRNA, tRNA	Single	Yes	7	Yes	25	Yes	100
12S, 16S	–	–	Single	No	–	No	–	Yes	66
tRNA	–	–	Single	No	–	No	–	No	–
			Partition scheme						
Protein coding genes	$1_N2_N3_N$	First, second, third codon positions	$1_N2_N3_N$	Yes	4	Yes	17	Yes	99
Protein coding genes	$1_N2_N3_{RY}$	First, second, third codon positions	$1_N2_N3_{RY}$	Yes	18	Yes	45	Yes	100
Protein coding genes	1_N2_N	First and second codon positions	1_N2_N	Yes	13	Yes	28	Yes	98
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_N$	First, second, third codon positions, rRNA, tRNA	$1_N2_N3_N$ rRNA tRNA	Yes	8	Yes	20	Yes	100
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_{RY}$	First, second, third codon positions, rRNA, tRNA	$1_N2_N3_{RY}$ rRNA tRNA	Yes	22	Yes	46	Yes	100
Protein coding genes, 12S, 16S, tRNA	1_N2_N	First and second codon positions, rRNA, tRNA	1_N2_N rRNA tRNA	No	–	No	–	Yes	100
12S, 16S, tRNA	–	–	rRNA tRNA	No	–	No	–	Yes	17
			PartitionFinder best partition scheme						
Protein coding genes	$1_N2_N3_N$	Protein coding genes	(atp6, atp8) (co1) (co2) (co3) (cytb) (nd1, nd4l, nd5) (nd2, nd3, nd4)	No	–	Yes	13	Yes	97
Protein coding genes	$1_N2_N3_{RY}$	Protein coding genes	(atp6, nd4) (atp8) (co1, co3) (co2, nd3, nd4l) (cytb, nd1) (nd2, nd5)	No	–	Yes	11	Yes	100
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_N$	Protein coding genes, 12S, 16S, tRNA	(atp6) (atp8, nd2, nd3, nd4) (co1) (co2) (co3) (cytb) (nd1, nd4l, nd5) (12S, 16S) (tRNA)	No	–	Yes	20	Yes	99
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_{RY}$	Protein coding genes, 12S, 16S, tRNA	(atp6, nd2, nd4, nd5) (atp8) (co1, co3) (co2, nd3, nd4l) (cytb, nd1) (12S, 16S) (tRNA)	No	–	Yes	21	Yes	100
Protein coding genes	$1_N2_N3_N$	Codon positions for protein coding genes	(atp61, nd11, nd41) (atp62, nd4l2, nd52) (atp63, atp83, co23, co33, nd4l3) (atp81, nd21, nd32, nd51) (atp82) (co11) (co12, co32) (co13) (co21, nd4l1) (co22) (co31, cytb1) (cytb2, nd12) (cytb3, nd53) (nd13, nd23, nd31, nd43) (nd22, nd33, nd42)	No	–	Yes	23	Yes	99
Protein coding genes	$1_N2_N3_{RY}$	Codon positions for protein coding genes	(atp61, nd11, nd41) (atp62, nd4l2, nd52) (atp63, atp83, cytb3, nd13, nd31, nd43, nd53) (atp81, nd21, nd32, nd51) (atp82) (co11) (co12, co32) (co13, co23, co33, nd23, nd4l3) (co21, nd4l1) (co22) (co31, cytb1) (cytb2, nd12) (nd22, nd33, nd42)	No	–	No	–	Yes	99
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_N$	Codon positions for protein coding genes, 12S, 16S, tRNA	(atp61, nd11, nd41) (atp62, nd4l2, nd52) (atp63, cytb3) (atp81, nd21, nd32, nd51) (atp82) (atp83, nd4l3) (co11)	Yes	8	Yes	9	Yes	99

(continued on next page)

Table 1 *(continued)*

Included data	Coding	Included partitions	Partition scheme	Pleuronectiformes		Pleuronectoidei		Clade L/Carangimorpha	
				Monophyletic	Support	Monophyletic	Support	Monophyletic	Support
Protein coding genes, 12S, 16S, tRNA	$1_N2_N3_{RY}$	Codon positions for protein coding genes, 12S, 16S, tRNA	(co12, co32) (co13) (co21, co31, cytb1, nd4l1) (co22) (co23, co33) (cytb2, nd12) (nd13, nd31, nd43, nd53) (nd22, nd33, nd42) (nd23) (12S, 16S) (tRNA) (atp61, atp81, nd11, nd41) (atp62, nd4l2, nd52) (atp63, atp83, co23, co33, cytb3, nd13, nd23, nd31, nd43, nd4l3, nd53) (atp82) (co11) (co12, co32) (co13) (co21, nd4l1) (co22) (co31, cytb1) (cytb2, nd12) (nd21, nd32, nd51) (nd22, nd33, nd42) (12S, 16S) (tRNA)	No	–	Yes	52	Yes	100

supported as well as its relationship to Paralichthyidae (*Cyclopsetta*). We find Scopthalmidae, Achiropsettidae, and Rhombosoleidae to form a grouping as well. Cynoglossidae and Soleidae have high support to be most closely related to each other. In results that include pleuronectiform monophyly, the *Psettodes*-pleuronectoid divergence is the most basal among flatfish inferred diversification events.

Application of BIC to select the optimum partition scheme from among single, subjective (i.e. by codon positions and RNA type) and objective partition schemes as indicated by PartitionFinder generally demonstrated that the subjective partition schemes were preferable (Table 2). For the $1_N2_N3_{RY}$RT dataset, the lowest BIC score was found for five partitions which supports pleuronectiform monophyly. The other four partitioning schemes analyzed for $1_N2_N3_{RY}$RT had one, seven, and fifteen partitions and higher BIC scores and did not indicate pleuronectiform monophyly. Differences between the lowest BIC and higher BICs were $\gg 10$, in strong support against higher BICs. $1_N2_N3_N$, 1_N2_N3, $1_N2_N3_N$RT and 1_N2_NRT generally exhibit the same pattern.

Results of SH tests did not find the constrained (Centropomidae + Pleuronectoidei) topologies to be significantly better or worse than the topologies containing a monophyletic Pleuronectiformes generated without topological constraints (Supplementary Table 3). In the case of $1_N2_N3_N$RT and 1_N2_NRT, the constrained (Centropomidae + Pleuronectoidei) topology has a higher likelihood, but not significantly so.

4. Discussion

Our analyses yielded weak and inconsistent evidence for pleuronectiform monophyly. Alternative alignments of tRNA and rRNA sites had noticeable influence on inferred pleuronectiform relationships, which we do not include in this study. Interestingly, even pleuronectoid monophyly was not consistently or highly supported statistically in our analyses. In contrast, studies of pleuronectiform monophyly using multi-locus nuclear data offer strong support for the Pleuronectoidei (Betancur-R. et al., 2013b; Campbell et al., 2013a). The discrepancy may be evidence of the different ability of nuclear and mitochondrial DNA sequences to preserve information from internode segments of different relative duration.

Partitioning appeared to have a strong affect on potential outcomes. If we assume the monophyly of flatfishes as a starting point then a pattern of under-, appropriate, and over-parameterization emerges in results from alternative analysis configurations (Tables 1, 2). However, the true relationships are rarely known in phylogenetic studies and we cannot use these results as a true evaluation of PartitionFinder's performance. Analyses of two of the six datasets consisting of only protein coding genes ($1_N2_N3_N$, $1_N2_N3_{RY}$, and 1_N2_N) or protein coding and RNA genes ($1_N2_N3_N$RT, $1_N2_N3_{RY}$RT, and 1_N2_NRT) produce evidence of pleuronectiform and pleuronectoid monophyly when unpartitioned. These are the 1_N2_N and 1_N2_NRT configurations with pleuronectiform bootstrap values of 16% and 7% respectively. Increasing parameterization by considering that each codon position, rRNA, and tRNA sites should be modeled with separate parameters results in more frequent recovery of the monophyletic Pleuronectiformes and Pleuronectoidei (found in results from five of these six datasets). However, the bootstrap support for pleuronectiform monophyly from 1_N2_N declined from 16% to 13% with two partitions, and 1_N2_NRT under four partitions does not support pleuronectiform monophyly. The results suggest that optimal partitioning for 1_N2_N and 1_N2_NRT datasets is a single partition. Increased parameterization was produced by PartitionFinder in datasets that include third codon position sites from protein coding genes. PartitionFinder always increased the total number of partitions over the subjective partitioning schemes, with poor success at recovering pleuronectiform monophyly (one instance, eighteen partitions, bootstrap support of 8%). For example, ML topologies from the $1_N2_N3_{RY}$RT coding scheme do not include a monophyletic Pleuronectiformes for unpartitioned and highly partitioned analyses (seven and fifteen partitions), but they do under a moderate partitioning scheme. Continuing with assumed pleuronectiform monophyly as outcome indicative of performance, PartitionFinder appears to over-parameterize this dataset and does not improve the results of phylogenetic inference. Our separate computations of BIC indicate that fewer partitions than those selected by PartitionFinder should be preferred. In the case when codon positions for each gene were separated for PartitionFinder, the partition routine could have potentially selected the subjective scheme, but it did not. Therefore we have two indications of PartitionFinder potentially over-partitioning our datasets — flatfish monophyly and BIC score (Table 2).

There is no strong evidence for or against pleuronectiform monophyly with existing nuclear sequence data (Campbell et al., 2014), and our results here arrive at the same conclusion. Only a few nuclear gene sequences yield a monophyletic Pleuronectiformes when evaluated separately (Betancur-R. et al., 2013b; Campbell et al., 2014). As indicated by Campbell et al. (2014), an inability to correctly infer gene trees and/or a high degree of incomplete lineage sorting present in the clade L fishes are likely affecting these phylogenetic inferences. A benefit of mitogenomes is that each data partition should support the same underlying tree (i.e., one haploid, non-recombining locus, one tree) boosting the amount of characters that can be appropriately included in a concatenated analysis. Furthermore, the effective population size of mitochondrial genomes is much smaller (1/4) than that of nuclear gene data, and mitogenomic data should not be affected by incomplete lineage sorting to the degree that nuclear loci are. The results we present do indicate that there is very little signal in mitochondrial genome data supporting pleuronectiform monophyly or the affinity of Psettodoidei to

Table 2
Comparison of total partition number, maximum likelihood score from RAxML version 8.00, Bayesian information criterion (BIC) and evidence of pleuronectiform monophyly from twenty of the analyses presented in Table 1. The number of parameters and sample sizes (alignment length) used in the BIC calculations are included. Lines in bold highlight the partition schemes with the lowest BIC. In the Partition notes column, the origin of the partitioning scheme is indicated. Single indicates no partitioning and subjective partitioning was based on underlying biological characteristics (i.e. codon position, rRNA, tRNA). For those analyses with partition schemes generated by PartitionFinder, if partitioning was allowed by protein coding gene or codon positions of protein coding genes that is also indicated along with the other partitions in those analyses.

Data	Number of partitions	ML optimization likelihood	Parameters	Sample size	BIC	Pleuronectiform monophyly?	Partition notes
$1_N2_N3_N$	1	−548,501.16	218	10,908	1,099,278.66	No	Single
$1_N2_N3_N$	**3**	**−534,137.33**	**654**	**10,908**	**1,075,103.73**	**Yes**	Subjective.
$1_N2_N3_N$	7	−547,485.92	1526	10,908	1,110,906.31	No	Included gene partitions.
$1_N2_N3_N$	15	−532,669.71	3270	10,908	1,099,484.70	No	Included codon partitions.
$1_N2_N3_{RY}$	1	−325,031.71	218	10,908	652,339.77	No	Single.
$1_N2_N3_{RY}$	**3**	**−319,950.74**	**654**	**10,908**	**646,730.53**	**Yes**	Subjective.
$1_N2_N3_{RY}$	6	−324,233.92	1308	10,908	662,125.96	No	Included gene partitions.
$1_N2_N3_{RY}$	13	−318,685.51	2834	10,908	666,963.60	No	Included codon partitions.
1_N2_N	1	−182,100.26	218	7272	366,388.48	Yes	Single.
1_N2_N	**2**	**−180,729.31**	**436**	**7272**	**365,834.54**	**Yes**	Subjective.
$1_N2_N3_N$ RT	1	−619,413.18	218	13,742	1,241,153.07	No	Single.
$1_N2_N3_N$ RT	**5**	**−603,648.50**	**1090**	**13,742**	**1,218,930.51**	**Yes**	Subjective.
$1_N2_N3_N$ RT	9	−616,681.83	1962	13,742	1,254,303.97	No	Included gene, 12S, 16S, and tRNA partitions.
$1_N2_N3_N$ RT	18	−602,154.65	3924	13,742	1,246,189.91	Yes	Included codon, 12S, 16S, and tRNA partitions.
$1_N2_N3_{RY}$ RT	1	−395,563.30	218	13,742	793,453.31	No	Single.
$1_N2_N3_{RY}$ RT	**5**	**−389,084.70**	**1090**	**13,742**	**789,802.90**	**Yes**	Subjective.
$1_N2_N3_{RY}$ RT	7	−393,466.02	1526	13,742	803,218.95	No	Included gene, 12S, 16S, and tRNA partitions.
$1_N2_N3_{RY}$ RT	15	−387,824.02	3270	13,742	810,548.55	No	Included codon, 12S, 16S, and tRNA partitions.
1_N2_N RT	**1**	**−251,306.31**	**218**	**10,106**	**504,872.33**	**Yes**	Included gene, 12S, 16S, and tRNA partitions.
1_N2_N RT	4	−249,085.68	872	10,106	507,210.18	No	Included codon, 12S, 16S, and tRNA partitions.

some other clade L lineage. This insight is in agreement with the evidence reported to date bearing on these issues. A history of clade L taxa characterized by short internode distance as a result of the rapid radiation of living lineages in the group would generate a low amount of phylogenetic signal with a high degree of homoplasy (or noise), and consequently inconsistent and weakly supported results.

5. Conclusions

Mitogenomic evidence does not provide strong evidence for flatfish monophyly, nor does it support an alternative placement for *Psettodes*. The highest support for Pleuronectiformes and Pleuronectoidei is 22% and 46% (bootstrap support) generated in the same analysis, neither of which can be considered strong statistical support. The extremely low support values for pleuronectiform monophyly when present are not acceptable for concluding in favor of mitogenomic support for Pleuronectiformes. It is intriguing that a group of fishes with such striking morphologies arguing in favor of its monophyly (i.e., bilateral asymmetry) should exhibit weak ambiguous support for monophyly from molecular data across data sources. Additional study of molecular evolution of clade L fishes and alternative sources of evidence should be pursued to help resolve the question of flatfish origins. In particular, methodologies that are designed to accommodate for incomplete lineage sorting can use Pleuronectiformes as a model system to explore the effects of highly discordant phylogenetic signal among loci as these methods have not been effective so far (Betancur-R. et al., 2013b). As molecular datasets continue to increase in size, it is important to avoid relying solely on analyses of concatenated alignments, which are known to obscure the underlying variation in phylogenetic signal.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.08.053.

Acknowledgments

M. A. C. wishes to thank his graduate committee members N. Takebayashi, D. Wolf and J. Rhodes for their helpful discussions and editorial comments in relevance to this manuscript. For providing tissue and access to vouchers we thank the Center for Molecular Biodiversity Research (NSMT). This study was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, Numbers 19207007 and 22370035, Japan Society for the Promotion of Science KAKENHI Grant Number 24770082, United States National Science Foundation Grant Number DEB0963767 and the Ministry of Science and Technology, Taiwan, Grant Number 101-2611-M-002-016-MY3.

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