Exon-capture data and locus screening provide new insights into the phylogeny of flatfishes (Pleuronectoidei)

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Abstract

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There is an extensive collection of literature on the taxonomy and phylogenetics of flatfishes (Pleuronectiformes) that extends over two centuries, but consensus on many of their evolutionary relationships remains elusive. Phylogenetic uncertainty stems from highly divergent results derived from morphological and genetic characters, and between various molecular datasets. Deciphering relationships is complicated by rapid diversification early in the Pleuronectiformes tree and an abundance of studies that incompletely and inconsistently sample taxa and genetic markers. We present phylogenies based on a genome-wide dataset (4,434 nuclear markers via exon-capture) and wide taxon sampling (86 species spanning 12 of 16 families) of the largest flatfish suborder (Pleuronectoidei). Nine different subsets of the data and two tree construction approaches (eighteen phylogenies in total) are remarkably consistent with other recent molecular phylogenies, and show strong support for the monophyly of all families included except Pleuronectidae. Analyses resolved a novel phylogenetic hypothesis for the family Rhombosoleidae as being within the Pleuronectoidea rather than the Soleoidea, and failed to support the subfamily Hippoglossinae as a monophyletic group. Our results were corroborated with evidence from previous phylogenetic studies to outline regions of persistent phylogenetic uncertainty and identify groups in need of further phylogenetic inference.

Key words: phylogenomics, systematics, soles, flounders, incomplete-lineage-sorting, target-enrichment

1. Introduction[3]

[3] Abbreviations:
ICZN = International Commission on Zoological Nomenclature

47 Large molecular phylogenetic datasets have rapidly become more available due to the
48 widespread use of next-generation sequencing technology coupled with target-capture methods
49 and through the growing number of supermatrices representing aggregated data from published
50 studies and sequence repositories. Both approaches have featured prominently in recent studies
51 of phylogenetic relationships of fishes, including a study with a very sparsely-sampled matrix of
52 27 genes for 11,638 taxa (Rabosky et al., 2018) and a dataset with 1,100 loci for 303 taxa
53 (Hughes et al., 2018). The proliferation of these types of large datasets in recent years has placed
54 renewed emphasis on long-standing questions in molecular phylogenetics regarding the
55 importance of taxon sampling versus gene sampling, completeness of matrices, marker selection,
56 detecting sources of systematic bias, and comparing methodological approaches of tree inference
57 (e.g. concatenation vs. multi-species coalescent approaches). All of these considerations are
58 important as we endeavor to unravel historically challenging phylogenetic relationships. These
59 issues are at the forefront of fish phylogenetics and are well exemplified in the flatfishes
60 (Pleuronectiformes) – a group that has a long history of changing classifications and muddled
61 phylogenetics relationships for the last 250 years.
62 The flatfishes are a diverse group of bilaterally asymmetrical fishes containing more than
63 800 species (812 species listed as valid in Eschmeyer's Catalogue of Fishes as of May 2021;
64 Fricke et al., 2021) classified in 16 families and two suborders, Psettodoidei and Pleuronectoidei.
65 The bilateral asymmetry of flatfishes is easily recognizable, as all species have both eyes located
66 on one side of the body as adults, an adaptation for life near the seafloor. Species are found

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UWFC = University of Washington Fish Collection
AFSC = Alaska Fisheries Science Center
NEFSC = Northeast Fisheries Science Center
CSIRO = The Commonwealth Scientific and Industrial Research Organisation
KU = University of Kansas
LSU = Louisiana State University

globally in a variety of habitats including tropical coral reefs, coastal freshwater, shallow nearshore marine bottoms, and continental slopes extending deeper than 2000m. Typically feeding near or on the benthos, flatfishes occupy a large range of trophic niches, and many species are valuable to commercial fisheries (Cooper and Chapleau, 1998a; Herrmann and Criddle, 2006; Wilderbuer et al., 2015). The morphological and taxonomic diversity found in flatfishes is well documented, but there is a high level of discordance among the characters that have been used in phylogenetic analysis, challenging our ability to test any one of the numerous phylogenetic hypotheses that have been proposed across a long and complex taxonomic history.

Early revisions to the flatfish taxonomy primarily aimed to incorporate newly described species into a rudimentary classification and were less focused with a holistic reevaluation of the overall existing taxonomy. In his System Naturae 10th ed., Linnaeus (1758) listed the sixteen flatfish species known to him all within one genus *Pleuronectes*. As more species were described, taxonomists began to identify morphologically distinct sub-groups and accounted for them by establishing new genera and higher-level taxa. In order to preserve a classification with all flatfish species under one taxonomic name, the flatfish taxon had to be expanded, first to family (Cuvier, 1816), then to the suborder Heterosomata (Cope, 1871; Gill, 1893; Jordan and Evermann, 1898), and eventually to the contemporary order Pleuronectiformes (Berg, 1940). Once the classification started to differentiate flatfishes at the family level, Pleuronectidae was reserved for only those fishes commonly referred to as flounders, with the soles and spiny turbots subsequently assigned their own families (Soleidae and Psettodidae, respectively; Jordan and Evermann, 1898; Regan, 1910). In these classifications, however, the morphology defining pleuronectid flounders is not clearly established and appears to represent a generalized flatfish body-plan, lacking the striking apomorphic features that define other families (reduced mouth,

90 head, eyes, and fins in the Soleidae, and spiny fins in Psettodidae). Consequently, any new
91 flatfish species lacking apomorphic features were placed in Pleuronectidae without holistic
92 reexamination of the taxonomy. Furthermore, the Pleuronectidae and Soleidae were again split
93 according to ocular orientation (Regan, 1910; Hubbs, 1945), having eyes on either the right side
94 (dextral) or left side (sinistral) of the body, a trait that while conspicuous, may not be as
95 phylogenetically informative as treated at the time. Thus, the Pleuronectidae became a "trash bin"
96 taxon and the overall flatfish classification began to accumulate architecture based on traits with
97 questionable phylogenetic information.
98 Early studies on flatfishes took place before the rise of phylogenetic systematics and
99 formal cladistic analyses. Nevertheless, figures depicting cladograms of the various flatfish
100 taxonomic groups can be found in early works such as Regan (1910) and Norman (1934).
101 Hensley and Ahlstrom (1984) and Ahlstrom et al. (1984) summarized the collective results from
102 Norman (1934, 1966), Hubbs (1945), and Amaoka (1969) in what is sometimes referred to as the
103 "Regan-Norman model" (Hensley, 1997; Berendzen and Dimmick, 2002). Hensley and
104 Ahlstrom (1984) found the Regan-Norman model to be poorly supported by existing
105 morphological evidence and listed a number of taxa they suspected were not monophyletic. In
106 response, Chapleau (1993) provided the first formal phylogenetic analysis of the flatfishes
107 constructed using a character state matrix of 39 morphological characters. His work was largely
108 accepted, and subsequent revisions mainly contributed by appending previously excluded
109 families and making minor adjustments (Cooper and Chapleau, 1998b; Evseenko, 2000; Hoshino
110 and Amaoka, 1998; Hoshino, 2001). Chapleau's model (1) resolved the "trash bin" family
111 Pleuronectidae (*sensu* Regan, 1910) into five likely monophyletic groups (Paralichthodidae,
112 Poecilopsettidae, Pleuronectidae, Rhombosoleidae, and Samaridae) that have largely remained

intact, (2) provided synapomorphies for taxa in need of more concrete definitions, and (3) established 14 of the 16 family-level groups recognized today. The relationships among these families continue to be debated, and new family group names are still only recently being recognized (Campbell et al., 2019).

Molecular phylogenies brought forth new uncertainties in the systematics of flatfishes. Relationships inferred from genetic data disputed those previously generated from morphological data, and with the inclusion of a wide sampling of outgroups, with several studies challenging the support for monophyly of the Pleuronectiformes as a whole. These studies found the problematic family Psettodidae, the only family in the suborder Psettodoidei, in other parts of the diverse acanthomorph clade Carangaria (Li et al., 2011; Near et al., 2013; Betancur-R et al., 2013a; Campbell et al., 2013; Shi et al., 2018, Lü et al., 2021), while others still supported a monophyletic Pleuronectiformes (Berendzen and Dimmick, 2002; Betancur-R et al., 2013b; Betancur-R and Ortí, 2014; Harrington et al., 2016). Aside from questions regarding monophyly of the entire flatfish clade, the cumulative results of many studies have shown that phylogenetic uncertainty is widespread throughout the flatfish tree. Discrepancies in the placement of Psettodidae as well as the relationships between many other flatfish clades may stem from inconsistencies between studies in taxon sample size, taxon representation, number of genetic markers used, and phylogenetic informativeness of the selected markers. The issue is further complicated by rapid diversification early in the flatfish tree (Ribeiro et al., 2018; Evans et al., 2021), potentially leading to incomplete lineage sorting (ILS), and extensive heterogeneity in the datasets in the form of both rate variation among lineages (heterotachy) and non-stationarity (base compositional heterogeneity), with many flatfish lineages having extremely long branches and extreme compositional biases (Betancur-R et al., 2013b).

With more than 800 species in the Pleuronectiformes, it is challenging to conduct an order-wide phylogenetic analysis with complete species coverage, so the studies thus far have inferred relationships at higher taxonomic levels or used taxon-rich sampling for specific subgroups, such as Pleuronectidae (Cooper and Chapleau, 1998a; Vinnikov et al., 2018), Rhombosoleidae (Guibord, 2003), and Scophthalmidae (Chanet, 2003). Byrne et al. (2018) provided the largest taxon-coverage of the order to date with 332 species and 9 genetic markers. Their analysis provided several novel topologies and found many polyphyletic taxa (notably the families Poecilopsettidae and Citharidae, and numerous genera), which contradict previous studies.

Disagreement among flatfish phylogenies may stem from different authors using different sets of genetic markers. For example, Byrne et al. (2018) sampled heavily from mitochondrial genes, which might explain differences from other works that primarily used nuclear loci. To overcome the issue of gene-specific bias, studies have gradually made a concerted effort in sampling more genetic data and from different regions of the genome. The largest flatfish dataset using Sanger sequencing (Sanger et al., 1977) was conducted by Betancur-R and Ortí (2014) who sampled 23 genetic markers and 85 species. Rabosky et al. (2018) featured 220 species in their supermatrix analysis, but their dataset was extremely sparse and was more focused on broader relationships across ray-finned fishes. The Betancur-R and Ortí (2014) analysis has been used to inform the contemporary classification scheme (Betancur-R et al., 2017) and validated the family designations proposed by Chapleau (1993). Harrington et al. (2016) and Lü et al., (2021) examined deep relationships within the Pleuronectiforms using genome-wide datasets derived from high-throughput “next-generation” sequencing methods. Harrington et al. (2016) examined the relationships of 19 species in 11 families using 1,314 loci of ultraconserved DNA

elements (UCEs) and found results that were highly consistent with that of Betancur-R et al. (2013b) and Betancur-R and Ortí (2014). Lü et al. (2021) used full genomes of 11 species in 9 families and found similar results but did not find a monophyletic Pleuronectiformes. Further application of next-generation methods to more taxonomically-rich datasets may allow flatfish systematists to attain more consistent results or identify sources of uncertainty.

Despite widespread disagreement among studies, comprehensive taxonomic and genetic sampling has resulted in some relationships being consistent. Recent analyses by Campbell et al. (2019), the only molecular phylogenetic study to include all 16 flatfish families to date, Betancur-R et al. (2013b), Betancur-R and Ortí (2014), and Harrington et al. (2016), converged on the same family-level topology, referred to in this study as the Betancur-Harrington-Campbell (BHC) model (Figure 1).

Figure 1: The BHC model of the flatfish tree based on phylogenies from Betancur-R et al. (2013b), Betancur-R and Ortí. (2014), Harrington et al. (2016), and Campbell et al. (2019). Ocular orientation categories are defined as such: 'Dextral' = all species within the family are dextral; 'Sinistral' = all species within the family are sinistral; or 'Mixed' = all species within the family have dextral and sinistral individuals (Psettodidae) or there is a mix of dextral species and sinistral species (Citharidae). Note: a small number of species in sinistral Paralichthyidae also have dextral individuals, and a small number of species in dextral Pleuronectidae also have sinistral individuals.

This study aims to improve our understanding of the relationships within flatfishes through the use of the most taxon-rich (86 vs. 11 and 19) next-generation dataset to date.

Specifically, we address issues of incomplete sampling at the family-level and within the family Pleuronectidae, and compare our results with the BHC model and other analyses to identify regions of persistent uncertainty within the phylogeny that are unlikely to be resolved with additional sampling. We use an exon-capture method developed by Li et al. (2013), which is a phylogenomic approach used to examine interspecies relationships across a wide range of evolutionary timescales, including at familial and ordinal levels in fishes (Kuang et al., 2018), but has never been applied to flatfishes. Our phylogenetic analyses of single-copy markers from pleuronectoid flatfishes are compared to results from other comprehensive molecular datasets – e.g., Betancur-R and Ortí (2014), Harrington et al. (2016), Byrne et al. (2018), Vinnikov et al. (2018). We also investigate the relative usefulness of filtering our loci based on molecular clocklikeness (MCL), nucleotide composition, and evolutionary rate.

2. Materials and methods

Exon sequence data were obtained for 96 specimens from two sources. A primary set of 57 samples were extracted from tissues, sequenced, and assembled here, and the remaining 39 samples were sourced from previously assembled data that were prepared as part of the FishLife project. The combined dataset represented 89 species (86 Pleuronectiformes and three outgroup species in the family Carangidae) and 12 family-level groups within the suborder Pleuronectoidei. Psettodidae is the only flatfish family not within the Pleuronectoidei and is not included in our dataset due to its contentious placement dictating the monophyletic status of Pleuronectiformes (Betancur-R et al., 2013a; Campbell et al., 2013; Shi et al., 2018, Lü et al., 2021) and the remaining Pleuronectoidei being a well-supported monophyletic group (Norman, 1934; Hensley and Ahlstrom, 1984; Chapleau, 1993; Berendzen and Dimmick, 2002; BHC model).

Cyclopsettidae (*sensu* Betancur-R et al., 2017) is not recognized as a valid family by Eschmeyer's Catalogue of Fishes (van der Laan and Fricke, 2021) as the name was not registered in ZooBank with its description (Campbell et al., 2019) as required by Article 8.5 of the ICZN, but there is strong evidence for its family-level status (Betancur-R et al., 2013b) and it will therefore be referred to as a distinct family-level group in this study. The term Cyclopsettidae was first used by Betancur-R et al. (2017), but this group has also been referred to as the Cyclopsetta group (Berendzen and Dimmick, 2002; Betancur-R et al., 2013b; Harrington et al., 2016), and Byrne (2018) asserts that Syaciumidae would be the appropriate family name of this group upon formal description according to the ICZN Principle of Priority.

The 57 samples prepared from frozen fin and muscle tissues were sub-sampled from specimens that were acquired from fish collections and trawl surveys conducted by the National Oceanic and Atmospheric Administration (NOAA). DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Genetic sequence data were obtained via the gene-capture protocol of Li et al. (2013). The method is appropriate for this study due to its reliable performance at recovering a large quantity of genomic data from non-model organisms across a range of evolutionary scales. The gene-capture method is able to resolve deep relationships and is sensitive enough to distinguish species level differences (Song et al., 2017; Li et al., 2018; Kuang et al., 2018). For this study we targeted 4,434 nuclear exons. This suite of markers was compiled from 17,817 putatively single-copy protein coding genes to include only those that capture efficiently across ray-finned fishes (Actinopterygii) (Jiang et al., 2019). A subset of 1,105 loci have been used for higher-level fish phylogenomics (Hughes et al., 2018, 2021). The size range of these markers was 102 to 5,803 bp with a mean size of 261 bp and a total concatenated alignment size of 1,157,304 bp.

These markers were derived from eight non-flatfish genomes, so we developed a custom set of baits based on a flatfish genome using the refinement step in Jiang et al. (2019) in attempts to increase sequence similarity between baits and target sequences, and thus increase capture efficiency. All 4,434 markers from Jiang et al. (2019) were blasted against a genome of *Pseudopleuronectes yokohamae* available on the National Center for Biotechnology Information library, GenBank (Genomic Resources Development Consortium, 2015; SAMD00021058) and the highest single-hit matches were used as the new targets. RNA baits and gene-capture reagents were supplied by the Arbor Biosciences myBaits Hybridization Capture Kit. Library preparation followed the protocol from Li et al. (2013) and target-capture hybridizations were done according to the myBaits Manual v.4.01 specifications, with baits diluted down as to use only 0.5μL per capture. Labwork was conducted at the University of Washington and Molecular Ecology Research Laboratory. The double capture method of Li et al. (2013) was used to increase concentrations of hybridized DNA. Sequencing was performed by the University of Delaware Sequencing and Genotyping Center on two lanes of Illumina HiSeq 2500 System using paired-end 150 bp reads.[4]

Raw reads were assembled into loci using the Assexon bioinformatics pipeline from Yuan et al. (2020). Read files were merged into one forward and reverse file per sample, then adapters and low-quality reads were trimmed using TrimGalore (Krüger, 2012). Duplicate sequences were removed and then the remaining reads were parsed to each locus by iteratively blasting against the reference markers. The sorted reads were then assembled into contigs iteratively for each gene and sample using String Graph Assembler (SGA) (Simpson and Durbin, 2012), then further assembled by the Assexon perl script merge.pl, utilizing alignment positions

[4] Raw sequence reads are available at the National Center for Biotechnology Information (NCBI) BioProject PRJNA684447.

249 generated by Exonerate (Slater and Birney, 2005). Potential paralogues were identified and
250 removed by finding the best reciprocal hits between assembled contigs and a reference genome
251 of *Oreochromis niloticus* with the perl script reblast.pl. A total of 4,431 of the 4,434 targeted
252 markers were captured by at least one sample, and all samples were represented by more than
253 2,000 loci. The assembled data were merged with 673 loci for 39 samples that were developed as
254 part of the FishLife project using a Carangaria-specific bait set and assembly pipeline (Hughes et
255 al., 2021). The combined dataset was aligned on amino acids using MAFFT (Katoh et al., 2002)
256 and translated back to codon-based alignment using the perl script mafft_aln.pl. After removing
257 poorly aligned markers, a total of 4,187 loci were used for downstream phylogenetic analysis.[5] A
258 summary of capture efficiency, data coverage, and other properties for each sample and locus
259 can be found in Table S1 and S2.
260 We reconstructed phylogenies using both concatenation-based and multispecies
261 coalescent (MSC) methods. In the concatenation-based approach, aligned genes for all taxa and
262 loci were combined into one supermatrix. Then using this master gene dataset, we estimated a
263 maximum likelihood (ML) tree using RAxML (Stamatakis, 2014) with 100 bootstrap (BS)
264 iterations under the GTRCAT model (suited for fast calculation and getting better likelihood
265 values if sample size is greater than 50, Stamatakis, 2006). In the MSC approach, we first
266 inferred ML trees for each gene alignment in RAxML using the same settings. Then, a MSC
267 phylogeny was inferred from all gene trees using ASTRAL-III (Zhang et al., 2018) and Local
268 Posterior Probability (PP) support was calculated (Sayyari and Siavash, 2016). Both
269 concatenation and MSC based methods were applied to our dataset consisting of all genes.
270 Eight additional phylogenetic analyses were conducted to investigate variability within
271 the data and stability of certain phylogenetic relationships (Table 1). For each treatment of the

[5] Alignments are available from Dryad (https://doi.org/10.5061/dryad.d51c5b036).

data, we used both concatenation-based and MSC-based approaches to construct phylogenies. The first treatment used a partition scheme based on first, second, and third codon-position. The second treatment inferred phylogenies based on amino acid sequences under the PROTCATJTT model. Two treatments aimed to reduce effects from missing data: one that removed loci with no data for at least 60% of the total number of taxa; the other retained only those loci that were present in both the FishLife dataset and the primary dataset. Filtering of genome-scale data is encouraged (Phillips et al., 2004; Townsend, 2007; Nosenko et al., 2013; Lopez-Giraldez et al., 2013), so for the remaining four treatments, we screened for loci based on MCL, nucleotide composition, and evolutionary rates (as inferred from average p-distance). The filter thresholds were in part determined with the aim of retaining roughly the same number of loci (~500) and amount of genetic material (~100,000 bp).

Table 1: Summary of datasets used in each treatment. aa = amino acids; SNP = single nucleotide polymorphism; PIS = phylogenetically informative site.

Dataset	Loci	Total size (bp)	Avg. size ± σ (bp)	Completeness (%)	SNP (%)	PIS (%)
1. Unfiltered	4187	940725	224.68 ± 213.35	44.45	33.26	21.17
2. Partitioned by codon	4187	940725	224.68 ± 213.35	44.45	33.26	21.17
3. Amino acid	655	145750 aa	74.89 ± 71.12 aa	40.42	36.72	19.68
4. Taxon coverage > 39	3051	598410	196.14 ± 107.73	58.32	37.81	25.47
5. FishLife overlap	663	216330	326.23 ± 186.80	67.48	41.65	29.11
6. clocklike	474	229866	484.95 ± 472.96	30.09	29.54	17.46
7. treeness/RCV > 13	875	174162	199.04 ± 131.81	51.76	32.10	21.68
8. p-dist < 0.05	551	105147	190.83 ± 140.89	47.09	25.97	14.07
9. p-dist > 0.08	617	161916	262.42 ± 214.58	43.04	40.25	27.70

A dataset of the most clocklike loci was generated to select for phylogenetically useful genes and remove those that may be contributing misleading phylogenetic information due to complex evolutionary histories or undetected paralogues (Kuang et al., 2018). The clocklikeness

290 test, which produces and compares likelihood values for a ML tree with and without a molecular
291 clock constraint for each gene, using a ratio between the values as a proxy for MCL. We found
292 the ratio of likelihood values calculated in PAUP (Swofford, 2003) to correlate with gene length,
293 making the test favor smaller genes with less information, so we followed Kuang et al. (2018)
294 and created a modified MCL by dividing the original ratio by the gene's length, clocklikeness
295 being represented by lower values representing. Our complete dataset was screened to include
296 only those genes with a modified MCL ratio less than 0.6 and length greater than 500bp. This
297 resulted in a filtered dataset consisting of 474 genes. The second filtered dataset was screened for
298 loci that were least susceptible to nucleotide composition bias based on the treeness to relative
299 composition variability (RCV) ratio used by Phillips and Penny (2003). Loci that are least likely
300 to be affected by such bias were those with homogenous base composition (small RCV) and
301 gene trees containing smaller branches nearer to the tips (high treeness), and therefore larger
302 treeness/RCV values. The filtered dataset was comprised of 875 loci with treeness/RCV values
303 greater than 13. Two datasets were screened based on evolutionary distance or p-distance to test
304 and optimize for the most parsimonious sites (Kuang et al., 2018) given the evolutionary scales
305 being inferred for the Pleuronectoidei. One dataset contained 551 conserved loci with average p-
306 distance smaller than 0.05, the other with 617 highly divergent loci with average p-distance
307 greater than 0.08.
308 The filter criteria, MCL, treeness/RCV, and p-distance, were evaluated for their
309 effectiveness in predicting the performance of each locus. For each criterion, we tested for a
310 correlation between that criterion's value and the tree-distance of each locus, tree-distance
311 calculated as the Branch Score Distance (Kuhner and Felsenstein, 1994) between the locus's
312 gene tree and a 'known' reference tree. In our case, we use the unfiltered-concatenation-based

313 tree (Figure 3) as an approximation of the reference tree, since true topology is unknown.
314 Another tree-distance metric, the Robinson-Foulds Distance (Robinson and Foulds, 1981) was
315 considered but not implemented as it compares topology only and does not account for branch
316 lengths. Gene trees with a low Branch Score Distance are similar to the reference tree and are
317 determined to be more phylogenetically informative. Specifically, we predict a positive
318 relationship between MCL and Branch Score Distance (i.e. clocklike genes with a low MCL
319 score would perform better than non-clocklike genes), and a negative relationship between
320 treeness/RCV and Branch Score Distance (i.e. genes with a high treeness/RCV score, indicating
321 low compositional bias, would perform better than genes with biased base composition). We did
322 not have any *a priori* expectations of a relationship between p-distance and Branch Score
323 Distance, as very fast-evolving genes could introduce homoplasy, but slow-evolving genes may
324 lack phylogenetically informative sites.
325 **3. Results**
326 The eighteen phylogenetic analyses resulted in trees with slightly different topologies and
327 varying levels of support (Table 2). Based on the 93 nodes that were evaluated, the partitioned-
328 concatenation-based tree had the highest overall support. The unfiltered, partitioned, and taxon-
329 coverage analyses had among the highest support, however, they all contained a few
330 relationships that were not widely supported when compared across analyses. Phylogenies
331 inferred from the conserved loci (p-distance < 0.05) were largely unsupported in both
332 concatenation-based and MSC-based methods and failed to resolve well-established
333 monophyletic groups (Figures S16 and S17), so this treatment is not included in further
334 comparative analysis.
335

Table 2: Summary of support for concatenation and MSC-based methods applied to each data treatment.

	Concatenation-based		MSC-based	
Dataset	**Avg. support (BS)**	**No. nodes with BS < 100**	**Avg. support (PP)**	**No. nodes with PP < 1**
1. Unfiltered	98.59	9	0.960	14
2. Partitioned by codon	99.00	6	0.949	13
3. Amino acid	95.11	20	0.885	32
4. Taxon coverage > 39	98.02	13	0.963	15
5. FishLife overlap	97.24	15	0.950	21
6. clocklike	94.53	21	0.903	33
7. treeness/RCV > 13	93.13	27	0.893	36
8. p-dist < 0.05	84.30	50	0.796	64
9. p-dist > 0.08	95.65	18	0.915	28

Two of the filtering parameters, p-distance and treeness/RCV, were poorly correlated with Branch Score Distance (Figure 2), indicating that on a gene-by-gene basis, faster/slower evolving or more/less heterogenous loci did not result in a tree closer to the reference tree (Figure 3) and that locus performance is not well predicted from these criteria. Analyses based on combining loci with highest p-distance, and those combining the highest treeness/RCV values, both resulted in moderately supported trees (Table 2) that contained relationships divergent from the reference tree. There was a significant correlation between MCL and Branch Score Distance, albeit with a poor fit ($R^2 = 0.31$), indicating that there may be utility in filtering out non-clocklike loci, however, neither family topology based on clocklike genes was fully concordant with that of the reference tree. The unfiltered and partitioned treatments were based on the same set of loci (Table 1) and the resulting phylogenies were largely congruent with one another given the same tree-construction method. Tree-construction method had a strong influence on resulting phylogenies. In particular, the most frequent and well-supported position of Poecilopsettidae is

exclusively found using ASTRAL, while that of Scophthalmidae is favored by the concatenation-based method (Table 3).

Figure 2: Relationships between three filter parameters (p-distance, MCL, and treeness/RCV) and Branch Score Distance between gene trees and the unfiltered-concatenation-based tree. Loci that were selected for the respective filtering analysis are highlighted in pink. Variable family topologies from concatenation-based analysis of each filter shown above. Full phylogenies can be found in Figures S12, S14, and S18.

Figure 3: Phylogeny generated from a total evidence dataset of 4,187 genes using the concatenation-based method. "Hippoglossinae" = Hippoglossinae (*sensu* Vinnikov et al., 2018) excluding *Clidoderma* and *Lyopsetta*. Exact support values can be found in Figure S2.

Figure 4: Phylogeny generated from a total evidence dataset of 4,187 genes using the MSC-based method. "Hippoglossinae" = Hippoglossinae (*sensu* Vinnikov et al., 2018) excluding *Clidoderma* and *Lyopsetta*. Exact support values can be found in Figure S3.

Table 3: Nodal support (BS in concatenation-based trees and % in MSC-based trees) across all analyses for selected phylogenetic relationships. '+' = Relationship supported at BS=100 or 100%. '-' = Relationship not present in tree. Treatment numbers are as follows: 1 = Unfiltered, 2 = Partitioned by codon, 3 = Amino acid, 4 = Taxon coverage > 39, 5 = FishLife overlap, 6 = clocklike, 7 = treeness/RCV > 13, 8 = p-dist < 0.05, 9 = p-dist > 0.08. 'well-supported' indicates groups or positions that were resolved with support ≥ 95 BS or 95% in more than one analysis.

375 "Hippoglossinae" = Hippoglossinae (*sensu* Vinnikov et al., 2018) excluding *Clidoderma* and

376 *Lyopsetta*.

	Concatenation-based Treatment								MSC-		
Relationship	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**
Monophyly of families and subfamilies (genera sampled)											
Citharidae (1/5)	+	+	+	+	+	+	+	+	+	+	+
Achiridae (3/6)	+	+	+	+	+	+	+	+	+	+	+
Samaridae (2/4)	+	+	+	+	+	+	+	+	+	+	+
Poecilopsettidae (1/3)	+	+	+	+	+	+	+	+	+	+	+
Cynoglossidae (2/3)	+	+	+	+	+	+	+	+	+	+	+
Soleidae (3/30)	+	+	+	+	+	+	99	+	+	+	+
Scophthalmidae (2/3)	+	+	+	+	+	+	+	+	+	+	+
Rhombosoleidae (6/8)	+	+	+	+	+	+	+	+	+	+	-
Cyclopsettidae (3/4)	+	+	+	+	+	+	+	+	+	+	+
Bothidae (6/20)	+	+	+	+	+	+	+	+	+	+	+
Paralichthyidae (5/10)	+	+	+	+	+	+	+	+	+	+	+
Pleuronectidae (23/24)	-	+	+	-	86	37	-	+	11	+	+
Pleuronectidae : Atheresthinae (1/1)	+	+	+	+	+	+	+	+	+	+	+
Pleuronectidae : Pleuronichthyinae (1/1)	+	+	+	+	+	+	+	+	+	+	+
Pleuronectidae : Hippoglossinae (6/6)	-	-	-	-	-	76	-	-	-	-	-
Pleuronectidae : Microstominae (2/2)	+	+	+	+	+	+	-	+	+	+	+
Pleuronectidae : Pleuronectinae (13/14)	+	+	+	+	+	+	+	+	+	+	+
All well-supported positions of families within Pleuronectoidei											
Citharidae sister to all other Pleuronectoidei	+	+	-	+	+	-	-	+	+	+	+
Scophthalmidae-Rhombosoleidae-Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae sister to Achiridae-Samaridae-Poecilopsettidae-Cynoglossidae-Soleidae	-	-	-	-	93	-	-	98	+	+	38
Cynoglossidae and Soleidae are sister	+	+	+	+	+	95	+	+	+	+	+
Poecilopsettidae sister to Cynoglossidae-Soleidae	+	+	+	+	+	93	+	+	-	-	-
Samaridae sister to Poecilopsettidae-Cynoglossidae-Soleidae (may not contain Poecilopsettidae)	-	-	+	-	74	+	-	+	64	58	32
Achiridae sister to Samaridae-Poecilopsettidae-Cynoglossidae-Soleidae (may not contain Poecilopsettidae)	-	-	97	-	+	+	-	+	70	10	69
Cyclopsettidae and Bothidae are sister	+	+	+	+	+	+	+	+	+	+	+
Paralichthyidae and Pleuronectidae are sister (Pleuronectidae may not be monophyletic)	+	+	+	+	+	+	+	+	+	+	+
Cyclopsettidae-Bothidae and Paralichthyidae-Pleuronectidae are sister	+	+	+	+	+	83	+	+	+	+	-
Rhombosoleidae and Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae are sister (Rhombosoleidae may not be monophyletic)	+	+	+	+	+	+	+	+	+	+	83
Scophthalmidae sister to Rhombosoleidae-Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae (may contain Poecilopsettidae)	-	-	-	-	93	-	-	97	99	+	87
All well-supported positions of subfamily-level taxa within Pleuronectidae											
Atheresthinae sister to Pleuronichthyinae-Microstominae-Hippoglossinae-Pleuronectinae	-	+	+	-	86	37	-	+	11	+	+
Pleuronichthyinae sister to Microstominae-Hippoglossinae-Pleuronectinae	+	+	+	+	+	66	93	+	+	+	75
Lyopsetta sister to "Hippoglossinae"-Microstominae-*Clidoderma*-Pleuronectinae	+	+	-	+	-	-	+	+	+	+	-
Microstominae sister to "Hippoglossinae"-*Clidoderma*-Pleuronectinae-*Lyopsetta*	-	-	+	-	+	-	-	-	-	-	-
"Hippoglossinae" and Microstominae are sister	-	-	-	-	-	-	-	-	99	+	-
Clidoderma sister to "Hippoglossinae"-Microstominae	-	-	-	-	-	-	-	-	+	-	-
Pleuronectinae sister to "Hippoglossinae"-Microstominae-*Clidoderma*	-	-	-	-	-	-	-	-	+	-	-
Clidoderma and Pleuronectidae are sister	+	+	97	+	-	-	+	-	-	0	-
Microstominae sister to *Clidoderma*-Pleuronectidae	98	+	-	84	-	-	-	-	-	-	-
"Hippoglossinae" sister to *Clidoderma*-Pleuronectinae-Microstominae	+	+	-	+	-	-	-	-	-	-	-
All well-supported groups of genera within Hippoglossinae											
Reinhardtius-Hippoglossus-Eopsetta-Verasper	+	+	42	96	-	-	-	63	+	+	-

Hippoglossus-Eopsetta-Verasper	-	-	-	-	63	-	-	-	+	-	98
Reinhardtius-Hippoglossus	+	-	98	96	-	+	-	67	-	-	-
Eopsetta-Verasper	+	-	-	97	-	31	38	31	-	-	-
All well-supported groups of genera within Pleuronectinae											
Liopsetta-Platichthys	+	+	91	+	99	+	96	+	+	+	-
Platichthys-Pleuronectes	-	-	-	-	-	-	-	-	-	-	+
Liopsetta-Platichthys-Pleuronectes	+	+	+	+	+	+	+	+	+	+	-
Liopsetta-Platichthys-Pleuronectes-Myzopsetta	+	+	+	+	+	+	71	99	99	97	+
Isopsetta-Parophrys	+	+	+	+	+	+	+	+	+	+	+
Lepidopsetta-Psettichthys	+	+	96	+	+	-	99	83	97	96	-
Isopsetta-Parophrys-Lepidopsetta-Psettichthys	+	+	+	+	+	+	+	+	+	+	+
Isopsetta-Parophrys-Lepidopsetta-Psettichthys-Liopsetta-Platichthys-Pleuronectes-Myzopsetta	+	+	+	+	+	+	97	93	+	+	94
Tribe Pleuronectini (=above and including *Pseudopleuronectes*)	+	+	+	+	+	+	+	+	+	+	99
Acanthopsetta-Cleisthenes	+	+	85	+	+	+	+	+	+	+	-
Acanthopsetta-Cleisthenes-Hippoglossoides	+	+	-	99	99	-	75	63	+	+	-
Tribe Hippoglossoidini (*Acanthopsetta-Cleisthenes-Hippoglossoides-Limanda*)	+	+	+	+	+	+	+	+	+	+	+

All families were resolved as strongly monophyletic (Table 3, Figure 5) with the exception of Pleuronectidae. Pleuronectidae was resolved as monophyletic in thirteen of sixteen phylogenies, and with support BS < 95 or PP < 0.95 in six. In the three cases where monophyly was not found the pleuronectid genus *Atheresthes* was sister to a clade containing both the Paralichthyidae (*sensu* Campbell et al., 2019) and the remaining Pleuronectidae genera or only the former, with mixed support (BS=16, 77, 95).

Figure 5: Preferred topological hypothesis of Pleuronectiformes families. Topology is based on the most frequent and highest supported relationships across sixteen phylogenetic analyses. Pie charts show proportion of analyses that contain the relationship with support ≥ 95 BS or 0.95 PP. Dotted branches represent lineages that were not sampled in this study, positions based on the BHC model.

The overall family-level topology varied between methods of tree inference and data treatments. Individual analyses failed to converge on a single topology, with eight distinct family

topologies resulting from the sixteen analyses compared. When families were examined separately, however, each was preferentially found in a single position that was generally robust to filtering and inference methods (Figure 5). The concatenation-based Fishlife-overlap and p-distance analyses were the only two analyses that exhibited the preferred topological hypothesis for all families.

Some parts of the family tree were more consistently supported than others. Citharidae was sister to all other Pleuronectoidei in nearly all topologies, and all analyses invariably resolved a monophyletic group consisting of five families: Rhombosoleidae, Cyclopsettidae, Bothidae, Paralichthyidae, and Pleuronectidae. Additionally, a second group consisted of the well-supported sister families Soleidae and Cynoglossidae, and with less strongly supported inclusion of Poecilopsettidae, Samaridae, and Achiridae (Table 3, Figure 5).

The phylogenetic positions of several families were unstable and varied across analyses. Poecilopsettidae was sister to the Soleidae-Cynoglossidae clade in all concatenation-based phylogenies (Table 3), but MSC-based analyses found the family in several other positions with low support (PP=0.1-0.89). In all MSC-based analyses and two concatenation-based analyses, Scophthalmidae was most frequently found as sister to the Rhombosoleidae-Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae clade (inclusive of Poecilopsettidae in the Fishlife-overlap and base-composition MSC-based analyses) (Table 3). In three of the concatenation-based trees, Scophthalmidae was sister to all other Pleuronectoidei excluding Citharidae (BS=52, 79, 98), and in three others it was sister to Citharidae (BS=49, 74, 94) but separated by deep branch lengths. Achiridae and Samaridae were found to be closely related to the Poecilopsettidae-Cynoglossidae-Soleidae clade in all analyses except the base-composition-MSC-based analyses, noting displacement of Poecilopsettidae in MSC-based analyses (Table 3).

The most frequent arrangement places Samaridae as sister to the Poecilopsetta-Cynoglossidae-Soleidae clade followed by Achiridae (Table 3). Alternatively, Achiridae and Samaridae were sister to one another in four concatenation-based analyses (BS=60, 65, 71, 91) with deep branch lengths.

Of the five Pleuronectidae subfamilies, four were strongly supported monophyletic groups, while Hippoglossinae was not supported (Table 3). Our analyses resolve Hippoglossinae as three distinct lineages: one each for the monotypic genera *Lyopsetta* and *Clidoderma*, and one containing the genera *Eopsetta*, *Verasper*, *Reinhardtius*, and *Hippoglossus* (Figure 6). All six genera were only found to be united using the clocklike treatment (BS=76 and PP=0.97). Relationships between the four genera of the third group (referred to as "Hippoglossinae" in quotations going forward) are dubious, as there were two conflicting topologies that were well-supported in multiple analyses; concatenation-based analyses favored two sister groups, *Eopsetta-Verasper* and *Reinhardtius-Hippoglossus* (Table 3, Figure 6), while ASTRAL tended to place *Verasper* as sister to *Hippoglossus-Eopsetta*. *Lyopsetta* most frequently diverges prior to the clade containing Microstominae, Pleuronectinae, "Hippoglossinae," and *Clidoderma* (Table 3). Concatenation-based and MSC-based analyses disagree on the order in which these four lineages arose, providing two alternate topologies (Figure 6, Table 3). Species relationships within the Pleuronectinae are consistent across analyses with few deviations (Table 3).

Figure 6: Preferred topological hypothesis of Pleuronectidae genera. Topology is based on the most frequent and highest supported relationships across sixteen phylogenetic analyses. Pie charts show proportion of analyses that contain the relationship with support ≥ 95 BS or 0.95 PP. Polytomy highlighted in red is best represented by two alternate topologies shown below. Dotted

439 branch indicates the position of *Dexistes* (not sampled in this study) based on Vinnikov et al.
440 (2018).
441
442 Taxon representation in other families was sparse, but relationships of species that were
443 sampled showed remarkable uniformity, with little variation from the unfiltered trees (Figures 3
444 and 4). In both Achiridae and Soleidae the same topology for the three genera sampled was
445 invariably supported. Paralichthyidae was represented by nine species that also had no difference
446 in topology in our analyses. Rhombosoleidae species formed a consistent arrangement with some
447 variation in the position of *Rhombosolea* and *Colistium*. The Cyclopsettidae species relationships
448 were stable with some variation in position of *Citharichthys stigmaeus*. Relationships of
449 Bothidae species were moderately stable with some variable topologies, particularly concerning
450 the position of *Psettina*.
451 Four genera were resolved as non-monophyletic: *Ammotretis* (Rhombosoleidae),
452 *Ancylopsetta* (Paralichthyidae), *Citharichthys* (Cyclopsettidae), and *Paralichthys*
453 (Paralichthyidae). Three genera were found as paraphyletic containing another genus:
454 *Ammotretis* (2 spp.) contained *Azygopus*, *Ancylopsetta* (2 spp.) contained *Gastropsetta*, and
455 *Citharichthys* (3 spp.) contained *Etropus*. The three MSC-based analyses that did result in a
456 monophyletic *Ammotretis* did so with poor support (PP=0.83) or by removing the genus from its
457 family. The three analyses that contained a monophyletic *Citharichthys* were poorly supported.
458 (PP=34; BS=82, 85). Non-monophyletic arrangements were found in all trees for *Ancylopsetta*
459 and *Paralichthys*.
460 **4. Discussion**

461 Disagreement between the many phylogenetic hypotheses that have been proposed can be
462 attributed to studies using datasets with a relatively small and variable set of genetic markers and
463 taxa. By using the exon-capture method we aimed to expand upon the previous genome-wide
464 datasets focusing on flatfishes (Harrington et al., 2016; Lü et al., 2021) to infer evolutionary
465 relationships with greater taxonomic coverage. Our analyses show that even with increased
466 taxon-sampling, many relationships within the Pleuronectoidei are largely driven by tree
467 construction method and which set of genetic data the methods are applied to.

468 **4.1. Treatments and Gene filtering**

469 One advantage of using genome-scale data such as exon-capture is the ability to screen
470 loci based on a number of characteristics that may provide more informative results. Locus
471 filtering, while typically recommended (Phillips et al., 2004; Townsend, 2007; Nosenko et al.,
472 2013; Lopez-Giraldez et al., 2013), may not always have desirable effects (Koch, 2021) and
473 which parameters provide favorable results depends on the dataset (Shen et al., 2016). We chose
474 to select subsets of our data that differed based on several criterion: missing data, clocklikeness,
475 base composition, and evolutionary rates.

476 The two missing-data filters, one based on taxon coverage and the other addressing
477 combination of unbalanced datasets, both performed similarly to the unfiltered analyses the used
478 the same respective tree construction method. Optimizing for completeness of a data matrix is
479 common practice in phylogenetic studies, but Wiens (2006) found that sources of systematic
480 error are more associated with characters that are included in a dataset rather than a those that are
481 not. Our results also showed no significant deleterious effects from missing data. In fact, the
482 overall support in our phylogenies appears to increase with number of loci and base pairs (Tables

1 and 2), indicating that larger genomic datasets or those that contain more informative sites, may provide better supported relationships.

Filtering for the most clocklike loci is a technique that aims to remove loci that have complex patterns of nucleotide evolution across taxa in the phylogeny (Doyle et al., 2015), which may signify several factors that could cause gene-tree species-tree discordance (i.e. strong selection on loci in a particular lineage, etc.). Screening for clocklike genes has been shown to be a helpful tool for datasets that have abundant phylogenetic uncertainty (Doyle et al., 2015, Kuang et al., 2018). Our clocklike phylogenies, however, were not well supported and contained many relationships not present in the preferred trees (Figure 5 and 6), despite a significant relationship between the clocklikeness of a single gene and tree-distance (Figure 2). Low support in the analysis of clocklike genes is likely the result of applying an excessively narrow filter. Despite correcting MCL to account for locus length and including longer loci, the resulting dataset still contained the fewest loci (474), highest missing data (70%), and among the fewest informative sites (18%).

Many phylogenetic construction methods assume that base composition is homogenous or stationary, so compositional heterogeneity among loci and taxa is often a source of systematic error (Collins et al., 2005; Rodriguez-Ezpeleta et al., 2007), and variability in composition has been linked to uncertainty within the flatfishes (Betancur-R et al., 2013b; Betancur-R and Ortí, 2014). We attempted to reduce bias due to composition by filtering based on the compositional variability metric established by Phillips and Penny (2003), but loci expected to be the least biased failed to correlate with tree-distance. Furthermore, both resulting phylogenies were among the least well supported and produced unique family topologies, again, possibly due to over-filtering.

506 The Pairwise-distance between any two sequences becomes larger with time since
507 divergence and increased mutation rate. We filtered based on fast evolving and slow evolving
508 loci, using average p-distance as a proxy for evolutionary rate (Kuang et al., 2018). Divergent
509 loci contain more data for inferring phylogenies but are more susceptible to noise from rapidly
510 evolving sites with complex histories of mutation. Conserved loci contain fewer informative sites
511 that are less susceptible to noise. The high p-distance dataset was well supported and generated
512 one of the two trees that aligned with the preferred family topology. The conserved (low p-
513 distance) dataset failed to produce a well-supported phylogeny. This indicates that there is
514 insufficient data within the conserved loci to infer relationships in the flatfish tree, despite a deep
515 history of more than 50 MYA (Ribeiro et al., 2018). Both datasets further suggest that larger and
516 more variable datasets may result in better resolved phylogenies.
517 In addition to treatments based on filtering, we applied a codon-based partition scheme,
518 and inference based on amino acid sequences. While the amino acid analyses performed
519 neutrally with regard to support and topology, the partitioned analyses were among the best
520 supported, again partly due to the large dataset.
521 **4.2. Concatenation and MSC methods**
522 With all data treatments, the concatenation approach resulted in more well-supported
523 relationships. Concatenation and MSC-based methods are both commonly used in phylogenetic
524 inference (Betancur-R and Ortí, 2014; Harrington et al., 2016; Kuang et al., 2018; Li et al, 2018)
525 but differences in how each treats the data will affect resulting trees differently. In the
526 concatenation approach, phylogeny is inferred directly from informative sites, so results are more
527 heavily influenced by longer and faster evolving genes. The MSC method used by ASTRAL is
528 expected to reduce gene-specific biases, especially from ILS (Liu et al, 2009; Tonini et al., 2015),

529 but because our dataset is comprised of mostly small genes (<300 bp), the resulting species-trees
530 have higher rates of uncertainty. This is because the MSC model assumes that the gene-trees
531 informing the species tree are known without error and small genes provide little information to
532 derive each gene-tree, especially those that are evolving slowly (Xi et al., 2015). This effect is
533 compounded by a reduced number of genes in the filtered datasets, even though some contained
534 larger genes on average (Table 1). In summary, the MSC-based relationships contain less error
535 due to gene-specific bias but at the expense of uncertainty from small genes.
536 Tree construction method had a substantial effect on some of the inferred relationships,
537 particularly concerning the Poecilopsettidae, Scophthalmidae, and subfamilies of the
538 Pleuronectidae. It is not entirely clear why one method would reliably result in a well-supported
539 relationship where the other method fails. Relationships favored by the concatenation-based
540 method may be informed by smaller genes, where gene trees would fail to repeatedly resolve the
541 topology. Relationships that were better resolved using the MSC-based method may be informed
542 by genes particularly susceptible to ILS, effects of which were reduced by ASTRAL.
543 **4.3. Family-level relationships of the Pleuronectiformes**
544 Previous examinations on the evolutionary history of flatfishes have produced conflicting
545 results based on different methods. Phylogenetic uncertainty in the flatfish tree is also
546 demonstrated by our phylogenetic analyses; however, we show that despite obtaining conflicting
547 results among individual analyses, collective comparison of those analyses suggest emerging
548 support for a single preferred topology.
549 While we aimed to provide a relatively dense sampling of flatfishes, four flatfish families
550 were not examined in this study: Psettodidae, Paralichthodidae, Oncopteridae, and
551 Achiropsettidae. Psettodidae is the only flatfish group excluded from the Pleuronectoidei

552 (Berendzen and Dimmick, 2002; Betancur-R et al., 2013b; Betancur-R and Ortí, 2014;
553 Harrington et al., 2016) and its placement within the broader Carangaria remains uncertain (Li et
554 al, 2011; Near et al., 2013; Betancur-R et al., 2013a; Campbell et al., 2013; Shi et al., 2018, Lü et
555 al., 2021). Campbell et al. (2019) found that Paralichthodidae, Oncopteridae, and
556 Achiropsettidae are more closely related to Rhombosoleidae than to any other family. Currently,
557 there is no strong counterevidence against a monophyletic Paralichthodidae-Oncopteridae-
558 Achiropsettidae-Rhombosoleidae clade.
559 The evolutionary relationships of the BHC model were almost entirely replicated in this
560 study, the only exception being the position of Rhombosoleidae. Our results strongly suggest that
561 Rhombosoleidae be part of the Pleuronectoidea rather than in Soleoidea (Figure 5). Difference in
562 phylogenetic placement may be attributable to Rhombosoleidae diverging from other sampled
563 families near the base of the flatfish tree, when it appears these fishes were undergoing rapid
564 diversification, so small methodological changes may dictate its placement among these early
565 lineages. Furthermore, the absence of closely related lineages in our dataset (Oncopteridae,
566 Achiropsettidae, and Paralichthodidae) make this family susceptible to long branch attraction
567 (LBA). We recommend further analysis with dense sampling from all four families to (1)
568 confirm the position and monophyly of this clade, and (2) determine if the whole clade should be
569 placed within Pleuronectoidea or just Rhombosolidae.
570 Even with genome-scale data and relatively dense taxon sampling, we acknowledge that
571 many relationships with the flatfish tree are still tenuous. Division of the Pleuronectiformes into
572 its two suborders Psettoidei (*sensu* Regan, 1910) and Pleuronectoidei (*sensu* Chapleau, 1993) is
573 widely established, but monophyly of the order has mixed support. Within the Pleuronectoidei,
574 the family Citharidae almost always resolves as sister to all other groups (BHC model; this

study). Campbell et al. (2019) refers to the Citharidae lineage as the superfamily Citharoidea. Our results also support this position, but strong support for a monophyly between its six species has been and remains elusive (Regan, 1910; Amaoka, 1972; Hensley and Ahlstrom, 1984; Aboussouan, 1988; Chapleau, 1993; Cooper and Chapleau, 1998a; Byrne et al., 2018; Shi et al., 2018), likely stemming from its species being morphologically and genetically disparate from one another following an initial period of rapid divergence (Chapleau, 1993; Campbell et al., 2019). Furthermore, other families are occasionally placed within this early branch of the tree such as Scophthalmidae (this study) and Achiridae (Byrne et al., 2018; Shi et al., 2018; Azevedo et al., 2008) with mixed support. This may be an artifact of LBA as all involved families diverged near the base of the Pleuronectoidei, which may have allowed for the accumulation of homoplasies. Studies that place Achiridae near the Citharidae sample heavily from the mitochondrial genome. The sister branch to the Citharidae leads to an unstable region of the phylogeny relating the remaining families. Relationships in this region change considerably between studies; some are well supported, but in most cases they are poorly supported and characterized by short branches, likely caused by rapid radiation early on in flatfish evolution. Emerging from this polytomy, there are five fairly well supported lineages: (1) Achiridae, (2) the Samaridae-Poecilopsettidae-Cynoglossidae-Soleidae clade, (3) the Paralichthodidae-Oncopteridae-Achiropsettidae-Rhombosoleidae clade, (4) Scophthalmidae, and (5) the Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae clade.

In addition to being sister to the Citharidae, the Achiridae has historically been found as sister to the Paralichthodidae-Oncopteridae-Achiropsettidae-Rhombosoleidae clade (BHC model) with moderate support, but we find Achiridae to be sister to the Samaridae-Poecilopsettidae-

Cynoglossidae-Soleidae clade. With impartial affinity to either group, its placement remains unknown beyond branching early on in the flatfish tree.

The clade containing the Samaridae, Poecilopsettidae, Cynoglossidae, and Soleidae has widely been supported in molecular phylogenies (BHC model; Chapleau, 1993; Byrne et al., 2018) and continues to be verified in our study, however the inclusion of Poecilopsettidae is slightly contentious. Poecilopsettidae has appeared in several other places in the broader Pleuronectiformes tree (Berendzen and Dimmick, 2002; Ji et al., 2016) but its placement within this group is the most widely supported across phylogenies (BHC model; Byrne et al., 2018; Shi et al., 2018). Byrne et al. (2018) was the first to suggest a polyphyletic Poecilopsettidae. In their phylogeny, one strongly supported group of *Poecilopsetta beanie* and *P. plinthus* appears as sister to the Soleidae-Cynoglossidae clade, while a second less supported group of *P. hawaiiensis*, *P. natalensis*, *P. praelonga*, and *Marleyella bicolorata* appears as sister to the Oncopteridae-Achiropsettidae-Rhombosoleidae clade. When compared to other studies, the first group represents the poecilopsettid lineage from previously mentioned studies (BHC model) and includes the genus *Nematops* (Campbell et al., 2019), however, the validity of a second lineage is dubious. While Byrne et al. (2018) reported *Poecilopsetta natalensis* within the second group, Shi et al. (2018) found the species in the group one position, within the complex formed by Cynoglossidae, Soleidae, and Samaridae. Additionally, our data produced a monophyletic Poecilopsettidae and sample species from both of the groups reported in Byrne et al. (2018). The first Poecilopsettidae group of from Byrne et al. (2018) could also have been influenced by the inclusion of a member of the Citharidae, *Citharoides macrolepidotus*, possibly from contamination or misidentification.

Studies that infer phylogenetic relationships of the Rhombosoleidae and its closely related families, Achiropsettidae, Oncopteridae, and Paralichthodidae, are sparse. While our study only samples from Rhombosoleidae, our methods place the family as sister to the Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae clade, a position not previously reported. The Rhombosoleidae-Achiropsettidae-Oncopteridae-Paralichthodidae complex is positioned closer to Achiridae and the Cynoglossidae-Soleidae clade in the BHC model. Additionally, further studies are needed to elucidate the relationships within Rhombosoleidae, particularly concerning the position and monophyletic status of *Azygopus*, *Colistium*, *Psammodiscus*, and *Taratretis*. This study is the only molecular phylogeny to our knowledge that contains *Azygopus*. Our analysis found the genus within a monophyletic Rhombosoleidae as the sister group to *Ammotretis rostratus*, which does not support Guibord's (2003) hypothesis of *Azygopus* being within Achiropsettidae. Campbell et al. (2019) refers to the group containing the nine families discussed so far as the superfamily Soleoidea. Our analysis does not support a monophyletic Soleoidea (*sensu* Campbell et al., 2019).

The monophyletic Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae group is well supported in nearly all molecular phylogenies (BHC model; Pardo et al., 2005; Shi et al., 2018; Byrne et al., 2018; this study). In most cases the Scophthalmidae has been placed as the sister group to this clade (BHC model; Chapleau, 1993). Campbell et al. (2019) refers to the group containing these five families as the superfamily Pleuronectoidea. Our results invariably insert Rhombosoleidae as more closely related to the former group (Table 3, Figures 3-5), casting doubt on a monophyletic Pleuronectoidea (*sensu* Campbell et al., 2019). The group containing all six of these families superficially resembles the "bothoid" group (*sensu* Hensley and Ahlstrom, 1984) defined as the Bothidae, Paralichthyidae (excluding *Tephrinectes* and *Thysanopsetta*, but

642 including the Cyclopsettidae), Pleuronectidae (*sensu* Norman, 1934, which includes
643 Rhombosoleidae, Samaridae, and Poecilopsettidae), and *Brachypleura* because they all share a
644 unique caudal skeleton. This group has largely been shown to not be monophyletic, but the
645 contemporary model of the flatfish phylogeny would suggest that the "bothoid" caudal skeleton
646 had appeared early on in the flatfish tree and was modified in several lineages such as in several
647 Citharidae, Achiridae, and the Soleidae-Cynoglossidae clade. In our phylogenies, the
648 Scophthalmidae originates near the base of the tree and occasionally appears closer to the
649 Citharidae, and datasets of primarily mitochondrial genes have produced topologies with
650 Scophthalmidae being the sister group to the Rhombosoleidae (Byrne et al., 2018; Shi et al.,
651 2018).

652 **4.4. Species relationships of the Pleuronectidae**

653 Pleuronectidae (*sensu* Chapleau and Keast, 1988) is the most densely sampled family in
654 our analysis (38 of 61 species in 23 of 24 genera). Numerous studies have examined
655 phylogenetic relationships within this family (Cooper and Chapleau, 1998a; Kartavtsev et al.,
656 2008a, 2008b; Roje, 2010; Kartavtsev et al., 2016; Ji et al., 2016; Vinnikov et al., 2018). Of these,
657 Vinnikov et al. (2018) provides the most comprehensive dataset to-date, sampling from 60 of 63
658 recognized species and using sequences from seven genes. Their study established the five
659 subfamilies currently recognized, with Atheresthinae originating at the base of the
660 Pleuronectidae tree, followed by Pleuronichthyinae, which is sister to the group containing
661 Microstominae, Hippoglossinae, and Pleuronectinae. The results from our study are consistent
662 with the overall topology found by Vinnikov et al. (2018) with a few exceptions. We show that
663 *Lyopsetta* and *Clidoderma* should not be included in the Hippoglossinae since this region of the
664 tree is poorly supported and fails to resolve in one singular topology (Figures 6). Vinnikov (2018)

665 justified a monophyletic Hippoglossinae by finding the group united in all gene trees, however,
666 this lineage has low posterior support in their concatenation-based tree. Furthermore, our
667 analysis indicates that getting further resolution on the relationships between *Lyopsetta,*
668 *Clidoderma,* the remaining Hippoglossinae, Microstominae, and Pleuronectidae is dubious.
669 Collective inconsistencies and poor node support from Vinnikov et al. (2018) and this study
670 suggests there may be additional persistent uncertainty among the remaining four
671 Hippoglossinae genera, in the Pleuronectinae between *Psettichthys*, *Lepidopsetta*, *Isopsetta*, and
672 *Parophrys*, and between *Liopsetta*, *Platichthys*, and *Pleuronectes*. Suzuki et al (2001) had
673 questioned the monophyly of Pleuronectidae on the basis of the placement of *Atheresthes*. Our
674 data show that the origin of the *Atheresthes* lineage is rooted near the most recent common
675 ancestor between Pleuronectidae and Paralichthyidae and that its position is sensitive to
676 phylogenetic methodology, but most studies report inclusion of *Atheresthes* in Pleuronectidae
677 (Betancur-R and Ortí, 2014; Vinnikov et al., 2018; Byrne et al., 2018). The unstable position of
678 the *Atheresthes* branch could be further explained by limited genetic data in Suzuki et al. (2001)
679 and very long branch lengths in this study.

680 **4.5. Remaining knowledge-gaps in the flatfish phylogeny**

681 There have been numerous order-level molecular phylogenies that have attempted to
682 resolve relationships between flatfish species (BHC model; Berendzen and Dimmick 2002;
683 Azevedo et al. 2008; Byrne et al., 2018; Shi et al., 2018; this study), all are subject to problems
684 of incomplete taxon sampling and discordant genetic information. While the general structure
685 between families is consistently replicated, the sister clade to the Citharidae contains unstable
686 relationships that are challenging to decipher even with use of genome-scale data. The molecular
687 signature that is driving this pattern likely arose due to a period of rapid genetic evolution early

on in the flatfish tree. In cases such as these, further systematic resolution is unlikely, and taxonomists should be conservative and apply rigorous systematic techniques before creating taxonomic names for groups that might be easily disassembled. Future systematic studies should aim to continue using large genome-wide datasets and prioritize underrepresented clades with dense taxon sampling. Problematic taxa that require further investigation are outlined in Table 4.

Table 4: Extent of knowledge and sampling for all Pleuronectiformes families in available literature; Counts for genera (Gen.) and species (Spp.) based on Catalogue of Fishes (Fricke et al., 2021; van der Laan and Fricke, 2021). Questionably monophyletic genera have been found as non-monophyletic or were suspected to be in the reference listed.

Family [Gen./Spp.]	**Within-family relationships largely resolved?**	**Questionably Monophyletic Genera (reference(s)); [fraction of polytypic genera]**	**Genera not included in any molecular phylogeny**
Achiridae [6/35]	Yes; generic relationships mostly resolved in Byrne et al. (2018)	*Trinectes* (Azevedo et al. 2008; Byrne et al., 2018); [1/5]	none
Achiropsettidae [4/4]	Yes; few species	none	*Pseudomancopsetta*
Bothidae [20/168]	No; most extensive analysis: Fukui (1997) and Byrne et al. (2018), but still largely unknown relationships	*Arnoglossus* (Byrne et al., 2018), *Bothus* (Byrne et al., 2018), *Chascanopsetta* (Byrne et al., 2018), *Crossorhombus* (Byrne et al., 2018), *Engyprosopon* (Byrne et al., 2018), *Psettina* (Byrne et al., 2018), *Laeops* (Byrne et al., 2018); [7/16]	*Perissias*, *Tosarhombus*
Citharidae [5/6]	Yes; few species but with highly divergent characters, monophyly justified in Hoshino (2001)	none	none
Cyclopsettidae [4/50]	No, need a focus on sorting out *Citharichthys-Etropus* complex; morphological analyses: Hensley and Ahlstrom (1984) and Khidir et al. (2005)	*Citharichthys* (Betancur, 2014; Byrne et al., 2018; this study), *Etropus* (Byrne et al., 2018); [2/4]	none
Cynoglossidae [3/162]	No, need a focus on monophyly of *Cynoglossus*; most extensive analysis: Cooper and Chapleau (1988)	*Cynoglossus* (Byrne et al., 2018); [1/3]	none
Oncopteridae [1/1]	Yes; monotypic	none	none
Paralichthyidae [10/59]	No; highly conflicting topologies among molecular phylogenies and with inconsistent taxon sampling	*Ancylopsetta* (this study), *Paralichthys* (Byrne et al., 2018; this study), *Pseudorhombus* (Byrne et al., 2018); [3/6]	none
Paralichthodidae [1/1]	Yes; monotypic	none	none
Pleuronectidae [24/63]	Yes; last comprehensive analysis (Vinnikov et al., 2018)	none	none (*Pleuronichthys ocellatus*, *Platichthys luscus*, and *P. solemdali* only species not included in any molecular phylogeny)
Poecilopsettidae [3/21]	No, need a focus on monophyly of Poecilopsettidae with broader taxon sampling	*Poecilopsetta* (Guibord 2003; Byrne et al., 2018); [1/3]	none

Psettodidae [1/3]	Yes; few species	none	none
Rhombosoleidae [8/20]	No, need a focus on placement of *Psammodiscus*, *Azygopus*, and monophyly of Rhombosoleidae; last comprehensive analysis: Guibord (2003)	*Ammotretis* (this study), *Colistium* (Guibord 2003); [2/5]	*Psammodiscus*, *Taratretis*
Samaridae [4/30]	No, need a focus on monophyly of *Samariscus*	*Samariscus* (Byrne et al., 2018); [1/3]	*Samaretta*
Scophthalmidae [3/9]	Yes; last comprehensive analysis: Chanet (2003)	none	none
Soleidae [30/180]	No; most extensive sampling from Byrne et al. (2018), but many unsampled genera remain	*Aseraggodes* (Byrne et al., 2018), *Pardachirus* (Byrne et al., 2018), *Pegusa* (Byrne et al., 2018), *Solea* (Byrne et al., 2018), *Zebrias* (Byrne et al., 2018); [5/19]	*Achiroides*, *Barbourichthys*, *Barnardichthys*, *Dexillus*, *Leptachirus*, *Liachirus*, *Paradicula*, *Phyllichthys*, *Rendahlia*, *Rhinosolea*, *Synclidopus*, *Typhlachirus*, *Vanstraelenia*

5. Conclusions

Our analysis of flatfish systematics using an exon-capture dataset with relatively dense taxon sampling was mostly consistent with the leading phylogenetic hypotheses for the Pleuronectiformes (BHC model) and the Pleuronectidae (Vinnikov et al., 2018). We report a novel position for the family Rhombosoleidae and show extremely poor support for the subfamily Hippoglossinae. Collective inference using sixteen phylogenetic analyses demonstrates the tenuous nature of several flatfish relationships at various evolutionary scales, and how certain relationships are favored by particular methods. By examining our data within the historical context of flatfish systematics we were able to identify several regions where phylogenetic uncertainty is likely to remain and outline groups that should be targeted for further study.

Acknowledgements

This work was funded by the NOAA Award NA15OAR4320063, Archival Storage and Dissemination of Data on Northeast Pacific Fish Eggs, Larvae, and Adults, and supported in part by the William W. and Dorothy T. Gilbert Ichthyology Research Fund. Sequence data from the FishLife project were funded by National Science Foundation grants DEB-1932759 and DEB-1929248 to RBR, and DEB-2015404 to DA. Molecular benchwork was assisted by Jennifer

716 Gardner, Sam Ghods, and Emily McFarland. We are grateful to the various fish collections and
717 NOAA facilities, and their staff, who were instrumental in acquiring tissue samples for this
718 project: Katherine Maslenikov from the UWFC, Alison Deary, Jay Orr, Duane Stevenson, &
719 Morgan Busby from the NOAA AFSC, John Galbraith and Jakub Kircun from NOAA NEFSC,
720 Graham Alastair from CSIRO, Leo Smith and Andrew Bentley from KU, and Prosanta
721 Chakrabarty and Seth Parker from LSU. Thank you to Bruce Kingham and the University of
722 Delaware Sequencing and Genotyping Center for their DNA sequencing services, and to the labs
723 of Steven Roberts, James and Lisa Seeb, and Adam Leche, for lending some of their resources
724 toward this project.

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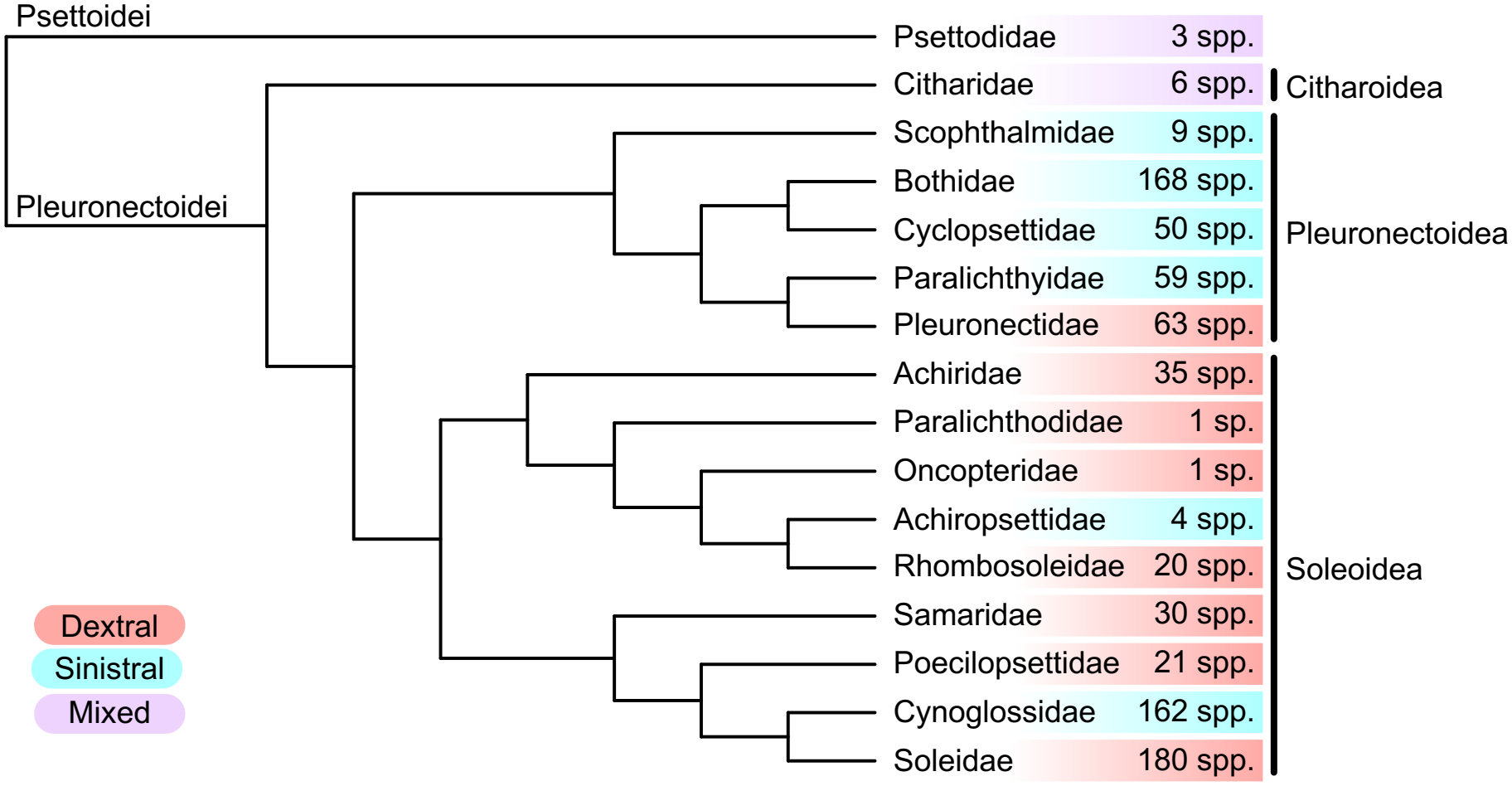
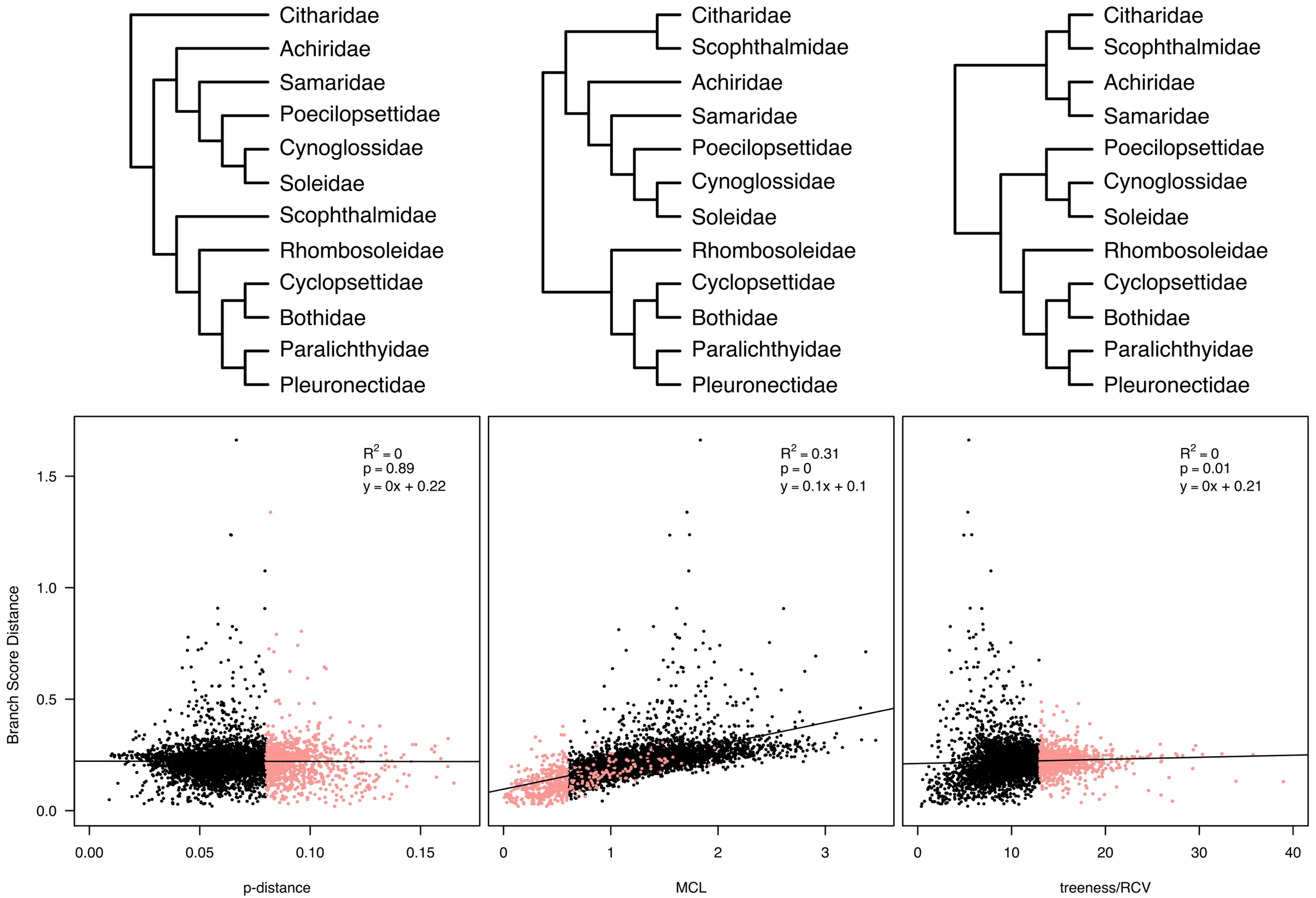

Psettoidei
Pleuronectoidei
Psettodidae 3 spp.
Citharidae 6 spp.
Citharoidea
Scophthalmidae 9 spp.
Bothidae 168 spp.
Cyclopsettidae 50 spp.
Paralichthyidae 59 spp.
Pleuronectidae 63 spp.
Pleuronectoidea
Achiridae 35 spp.
Paralichthodidae 1 sp.
Oncopteridae 1 sp.
Achiropsettidae 4 spp.
Rhombosoleidae 20 spp.
Samaridae 30 spp.
Poecilopsettidae 21 spp.
Cynoglossidae 162 spp.
Soleidae 180 spp.
Soleoidea
Dextral
Sinistral
Mixed


Citharidae
Achiridae
Samaridae
Poecilopsettidae
Cynoglossidae
Soleidae
Scophthalmidae
Rhombosoleidae
Cyclopsettidae
Bothidae
Paralichthyidae
Pleuronectidae
Citharidae
Scophthalmidae
Achiridae
Samaridae
Poecilopsettidae
Cynoglossidae
Soleidae
Rhombosoleidae
Cyclopsettidae
Bothidae
Paralichthyidae
Pleuronectidae
Citharidae
Scophthalmidae
Achiridae
Samaridae
Poecilopsettidae
Cynoglossidae
Soleidae
Rhombosoleidae
Cyclopsettidae
Bothidae
Paralichthyidae
Pleuronectidae
$R^2 = 0$
p = 0.89
y = 0x + 0.22
$R^2 = 0.31$
p = 0
y = 0.1x + 0.1
$R^2 = 0$
p = 0.01
y = 0x + 0.21
Branch Score Distance
0.0
0.5
1.0
1.5
0.00
0.05
0.10
0.15
p-distance
0
1
2
3
MCL
0
10
20
30
40
treeness/RCV


0.05
outgroup = Carangidae
Trachurus symmetricus UW156101
Carangoides plagiotaenia KU7154
Caranx ignobilis KU4947
Citharoides macrolepidotus KU2466
Citharoides macrolepidotus KU2468
Citharidae
Lepidorhombus boscii KU3496
Scophthalmus aquosus KU1252
Scophthalmidae
Plagiopsetta glossa KU2474
Samariscus japonicus KU2469
Samaridae
Gymnachirus texae KU3932
Achirus mazatlanus STRIBFT04923
Trinectes maculatus KU1501
Achiridae
Poecilopsetta plinthus KU2472
Poecilopsetta praelonga H6574–13
Poecilopsettidae
Cynoglossus interruptus KU2478
Symphurus diomedeanus KU5147
Symphurus plagiusa KU1061
Cynoglossidae
Aseraggodes heemstrai KU4996
Heteromycteris japonicus KU2492
Solea solea KU1846
Soleidae
Ammotretis lituratus H7106–00
Ammotretis rostratus H7106–06
Azygopus pinnifasciatus H7725–11
Colistium guntheri H5625–01
Rhombosolea plebeia H5924–01
Pelotretis flavilatus H5546–01
Peltorhamphus novaezeelandiae H5545–01
Rhombosoleidae
Syacium gunteri KU5095
Citharichthys stigmaeus UW155841
Citharichthys sordidus UW156520
Citharichthys xanthostigma UW153456
Etropus cyclosquamus KU5241
Etropus microstomus KU1506
Cyclopsettidae
Laeops kitaharae KU2505
Psettina tosana KU2507
Bothus lunatus KU154
Bothus pantherinus KU5642
Engyophrys senta KU5128
Crossorhombus kobensis KU2489
Engyprosopon multisquama KU2503
Bothidae
Atheresthes evermanni UW119477
Atheresthes stomias UW151948
Atheresthinae (Pleuronectidae)
Pseudorhombus pentophthalmus KU2481
Tarphops oligolepis KU2498
Paralichthyidae
Paralichthys californicus UW156295
Paralichthys dentatus KU1446
Paralichthys squamilentus KU5205
Paralichthys oblongus KU1493
Ancylopsetta dilecta KU5129
Ancylopsetta ommata KU10
Gastropsetta frontalis KU5223
Pleuronichthys guttulata KU484
Pleuronichthys coenosus UW151113
Pleuronichthys decurrens UW116910
Pleuronichthyinae
Lyopsetta exilis KU3176
Lyopsetta exilis UW155009
Lyopsetta
Eopsetta jordani KU555
Verasper moseri UW118095
Verasper variegatus UW117963
Reinhardtius hippoglossoides UW152953
Hippoglossus hippoglossus KU5417
Hippoglossus stenolepis UW150612
"Hippoglossinae"
Glyptocephalus cynoglossus KU1474
Glyptocephalus zachirus UW151776
Microstomus bathybius KU2269
Microstomus pacificus KU3209
Microstomus pacificus UW157426
Microstominae
Clidoderma asperrimum UW153538
Clidoderma
Limanda aspera UW151440
Limanda limanda KU5418
Limanda sakhalinensis UW152387
Limanda sakhalinensis UW157053
Hippoglossoides robustus UW119499
Hippoglossoides robustus UW154433
Hippoglossoides elassodon UW047660
Hippoglossoides elassodon UW156848
Hippoglossoides platessoides NEFSC90475
Hippoglossoides platessoides KU368
Hippoglossoides platessoides NEFSC95054
Acanthopsetta nadeshnyi UW118829
Cleisthenes herzensteini UW118821
Hippoglossidini
Pseudopleuronectes americanus KU365
Isopsetta isolepis UW156526
Parophrys vetulus UW156525
Psettichthys melanostictus UW154235
Lepidopsetta bilineata UW110234
Lepidopsetta bilineata UW112276
Lepidopsetta polyxystra UW125587
Lepidopsetta polyxystra UW150524
Myzopsetta ferruginea KU1028
Myzopsetta proboscidea UW117294
Pleuronectes platessa KU1845
Pleuronectes quadrituberculatus UW151438
Platichthys stellatus UW156364
Liopsetta glacialis UW150678
Liopsetta pinnifasciata UW44722
Pleuronectini
Pleuronectinae
Pleuronectidae
Bootstrap Support Values
100
95 - 99
80 - 94
< 80


1
Trachurus symmetricus UW156101
Carangoides plagiotaenia KU7154
Caranx ignobilis KU4947
outgroup = Carangidae
Citharoides macrolepidotus KU2466
Citharoides macrolepidotus KU2468
Citharidae
Poecilopsetta plinthus KU2472
Poecilopsetta praelonga H6574–13
Poecilopsettidae
Gymnachirus texae KU3932
Achirus mazatlanus STRIBFT04923
Trinectes maculatus KU1501
Achiridae
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Samariscus japonicus KU2469
Samaridae
Cynoglossus interruptus KU2478
Symphurus diomedeanus KU5147
Symphurus plagiusa KU1061
Cynoglossidae
Aseraggodes heemstrai KU4996
Heteromycteris japonicus KU2492
Solea solea KU1846
Soleidae
Lepidorhombus boscii KU3496
Scophthalmus aquosus KU1252
Scophthalmidae
Ammotretis lituratus H7106–00
Ammotretis rostratus H7106–06
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Colistium guntheri H5625–01
Rhombosolea plebeia H5924–01
Pelotretis flavilatus H5546–01
Peltorhamphus novaezeelandiae H5545–01
Rhombosoleidae
Syacium gunteri KU5095
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Citharichthys sordidus UW156520
Citharichthys xanthostigma UW153456
Etropus cyclosquamus KU5241
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Cyclopsettidae
Laeops kitaharae KU2505
Psettina tosana KU2507
Bothus lunatus KU154
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Engyophrys senta KU5128
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Bothidae
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Ancylopsetta dilecta KU5129
Ancylopsetta ommata KU10
Gastropsetta frontalis KU5223
Paralichthyidae
Atheresthes evermanni UW119477
Atheresthes stomias UW151948
Atheresthinae
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Pleuronichthys coenosus UW151113
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Pleuronichthyinae
Lyopsetta exilis KU3176
Lyopsetta exilis UW155009
Lyopsetta
Clidoderma asperrimum UW153538
Clidoderma
Glyptocephalus cynoglossus KU1474
Glyptocephalus zachirus UW151776
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Microstomus pacificus KU3209
Microstomus pacificus UW157426
Microstominae
Reinhardtius hippoglossoides UW152953
Verasper moseri UW118095
Verasper variegatus UW117963
Eopsetta jordani KU555
Hippoglossus hippoglossus KU5417
Hippoglossus stenolepis UW150612
“Hippoglossinae”
Pleuronectidae
Limanda limanda KU5418
Limanda aspera UW151440
Limanda sakhalinensis UW152387
Limanda sakhalinensis UW157053
Acanthopsetta nadeshnyi UW118829
Cleisthenes herzensteini UW118821
Hippoglossoides robustus UW119499
Hippoglossoides robustus UW154433
Hippoglossoides platessoides KU368
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Hippoglossoides elassodon UW156848
Hippoglossoidini
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Isopsetta isolepis UW156526
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Lepidopsetta bilineata UW112276
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Lepidopsetta polyxystra UW150524
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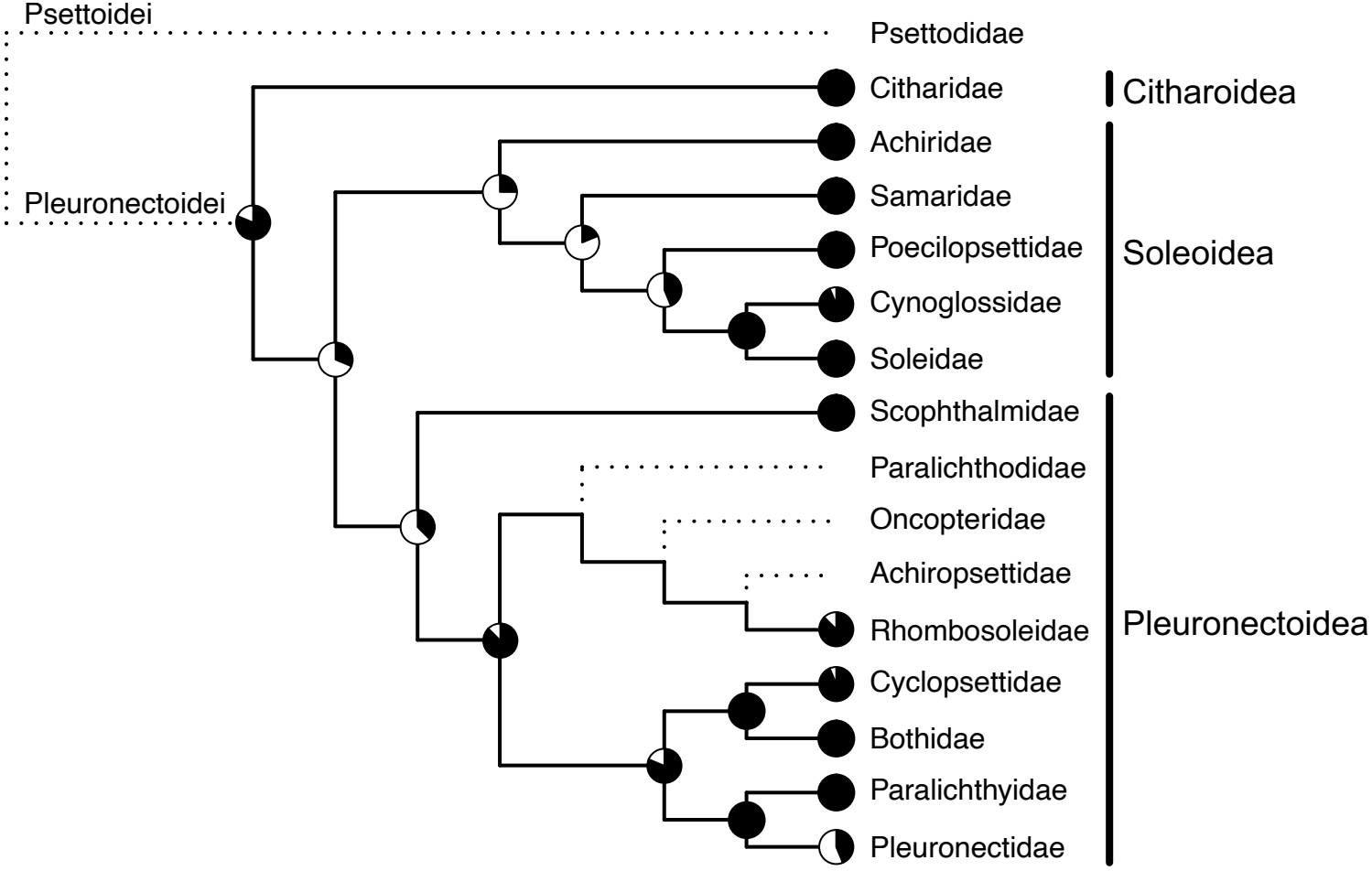
Pleuronectes quadrituberculatus UW151438
Platichthys stellatus UW156364
Liopsetta glacialis UW150678
Liopsetta pinnifasciata UW44722
Pleuronectini
Pleuronectinae
Local Posterior Probability
1
0.95 - 0.99
0.80 - 0.94
< 0.80


Psettoidei
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Paralichthodidae
Oncopteridae
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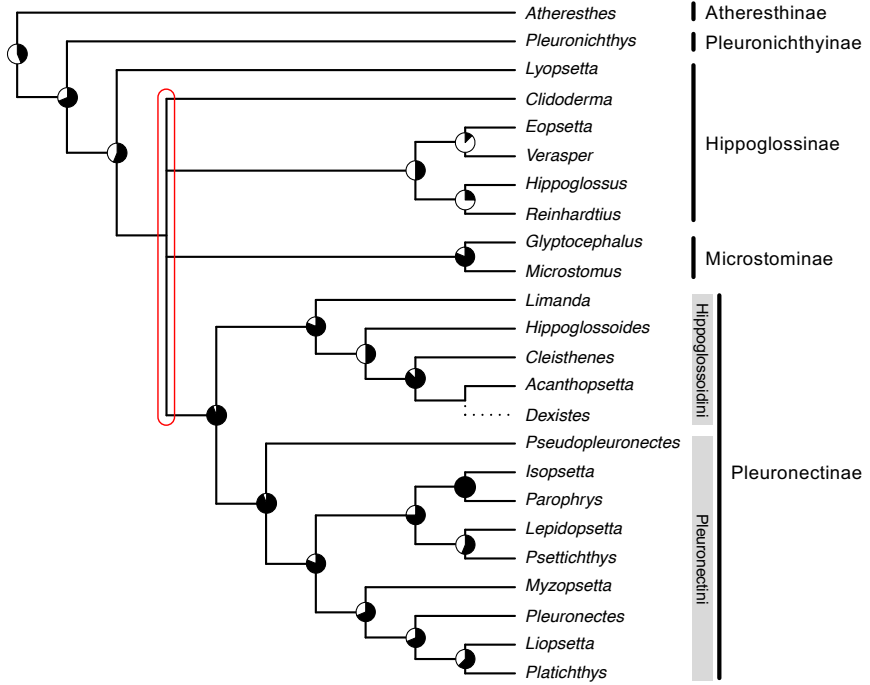
Soleoidea
Pleuronectoidea



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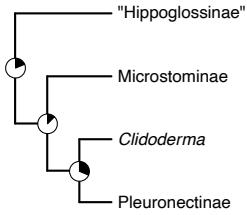
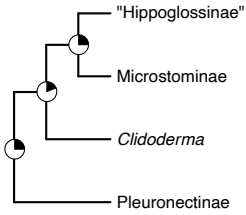
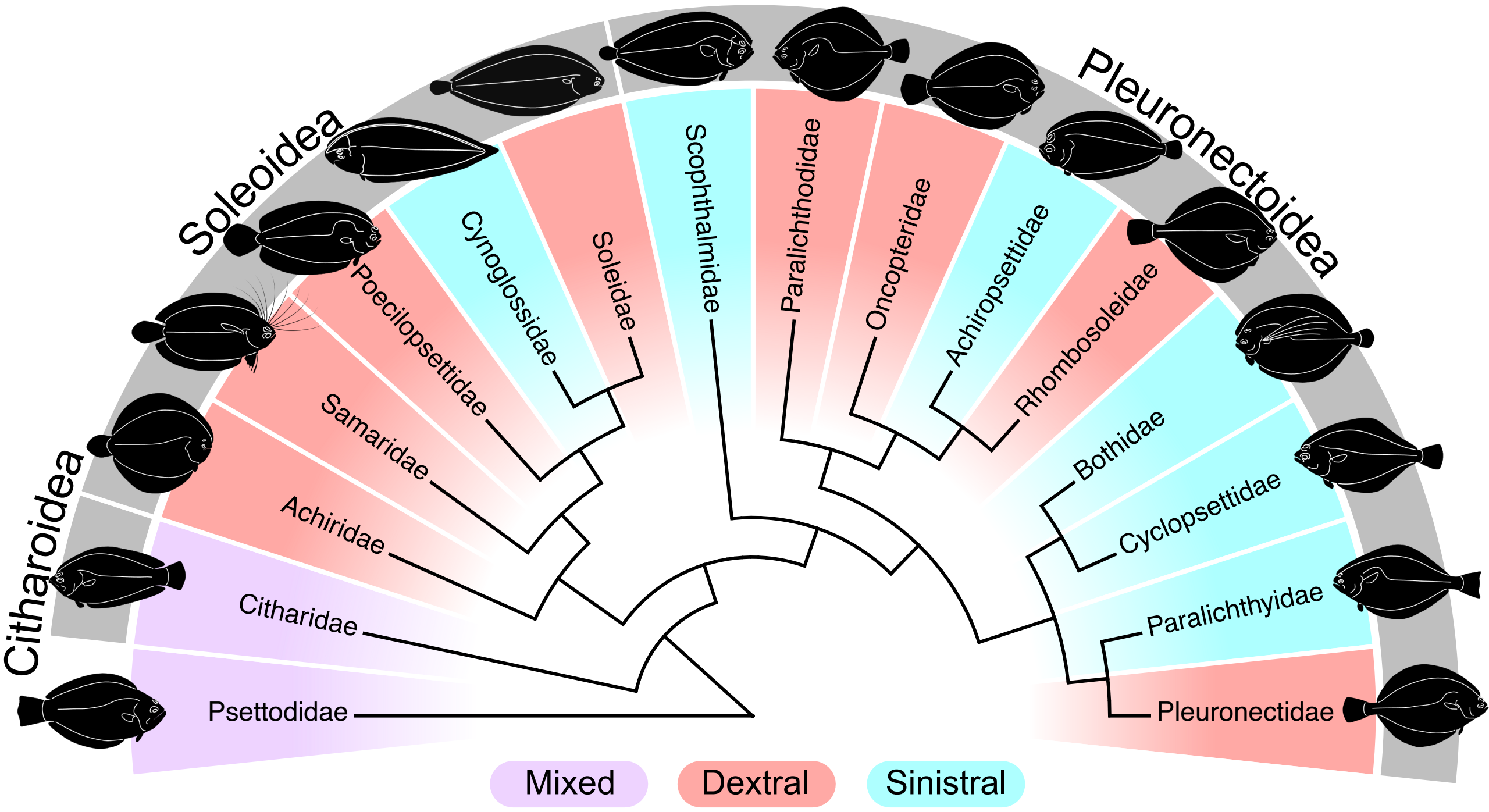


Resulted from analyses:
Unfiltered : Concatenation-based
Taxon coverage > 39 : Concatenation-based
Partitioned by codon : Concatenation-based

Topology 2



Resulted from analyses:
Unfiltered : Species-tree
Taxon coverage > 39 : Species-tree
treeness/RCV > 13 : Species-tree


Soleoidea
Pleuronectoidea
Citharoidea
Poecilopsettidae
Cynoglossidae
Soleidae
Scophthalmidae
Paralichthodidae
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Achiropsettidae
Rhombosoleidae
Samaridae
Achiridae
Bothidae
Cyclopsettidae
Citharidae
Paralichthyidae
Psettodidae
Pleuronectidae
Mixed
Dextral
Sinistral