An exploration of speciation and genome-wide uncertainty in flatfishes based on exon-capture data

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Abstract

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The flatfishes (Pleuronectiformes) have undergone numerous taxonomic revisions but many of their interrelationships still lack consensus among the scientific community at both the species-level and deep evolutionary timescales. Phylogenetic uncertainty stems from apparent bursts of evolution in short time periods early in the Pleuronectiformes tree, and this uncertainly is exacerbated by an abundance of studies that incompletely and inconsistently sample taxa and genetic markers. In order to partly address the issue of insufficient phylogenetic information, we inferred phylogenies based on the largest molecular dataset (4434 markers via exon-capture) and densest taxon sampling (86 species spanning 11 of the 15 families) of any flatfish study using next-generation sequencing to date. We compared our results to phylogenetic hypotheses across recent works, report several new hypotheses, and identify regions of the flatfish tree that are unlikely to be further resolved with additional sampling. We also conducted species delimitation analyses on four economically valuable species pairs in the family Pleuronectidae to evaluate their utility in being effectively managed. Species pairs in the genera *Lepidopsetta* and *Limanda* were found to be good species for management, while species pairs in *Hippoglossoides* and *Atheresthes* require further taxonomic analysis.

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General Introduction

The flatfishes (Pleuronectiformes) are a diverse group of bilaterally asymmetrical fishes containing more than 800 species (819 species listed as valid in Eschmeyer's Catalogue of Fishes as of September 2020; Fricke et al. 2020) classified in 16 families and two suborders (Psettodoidei and Pleuronectoidei). Species are found globally in a variety of habitats including tropical coral reefs, coastal freshwater, shallow nearshore marine bottoms, and continental slopes extending deeper than 1000m. Typically feeding near or on the benthos, flatfishes occupy a large range of trophic niches across species, and many species are valuable to commercial fisheries (Cooper & Chapleau 1998a, Herrmann & 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.

This study aims to contribute to our understanding of the evolutionary history of flatfishes by (1) using a next-generation molecular dataset to address issues of incomplete sampling at both the species-level and at deep evolutionary timescales, and (2) outlining the most effective steps to progress toward a more stable flatfish phylogeny and classification. In order to evaluate our novel results comparatively and constructively with previous hypotheses found across an extensive body of literature, we first established the taxonomic status and history of the Pleuronectiformes and each of its families.

Taxonomic and systematic history of the Pleuronectiformes

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 & Evermann 1989), and eventually to the contemporary order Pleuronectiformes (Berg 1940). Once the classification started to differentiate flatfishes at the family level, the 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 & 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 present in other groups (reduced mouth, head, eyes, and fins in the Soleidae, and spiny fins in Psettodidae). Consequently, any new flatfish species lacking apomorphic features were placed in Pleuronectidae without holistic reexamination of the taxonomy. Furthermore, the Pleuronectidae and Soleidae were again split according to ocular orientation (Regan 1910, Hubbs 1945), having eyes on either the right side (dextral) of left side (sinistral) of the body, a trait that while conspicuous, may not be as phylogenetically informative as treated at the time. Thus, the Pleuronectidae became a “trash bin” taxon and the overall flatfish classification began to accumulate architecture based on traits with questionable phylogenetic information.

The aforementioned early studies on flatfishes took place before the rise of phylogenetic systematics and formal cladistic analyses. Nevertheless, figures depicting cladograms of the various flatfish taxonomic groups can be found in early works such as Regan (1910) and Norman (1934). Hensley & Ahlstrom (1984) and Ahlstrom et al. (1984) summarized the collective results from Norman (1934, 1966), Hubbs (1945), and Amaoka (1969) in what is sometimes referred to as the "Regan-Norman model" (Hensley 1997, Berendzen & Dimmick 2002). Hensley & Ahlstrom (1984) found the Regan-Norman model to be poorly supported by existing morphological evidence and listed a number of taxa they suspected were not monophyletic. In response, Chapleau (1993) provided the first formal phylogenetic analysis of the flatfishes constructed using a character state matrix of 39 morphological characters. His work was largely accepted and subsequent revisions mainly contributed by appending previously excluded families and making minor adjustments; Cooper & Chapleau (1998b) added Paralichthodidae, Evseenko (2000) added Achiropsettidae, Cooper & Chapleau (1998b) resolved a large polytomy, Hoshino & Amaoka (1998) and Hoshino (2001) explored placement of the genus *Tephrinectes*, and Hoshino (2001) argued for a monophyletic Citharidae. Chapleau's model (1) resolved the "trash bin" family Pleuronectidae (*sensu* Regan 1910) into five likely monophyletic groups (Paralichthodidae, 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 continues to be debated, and new family group names are still only recently being recognized. For example, the families Oncopteridae and Cyclopsettidae were formally defined in the recent analysis by 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, several studies failed to provide support for monophyly of the Pleuronectiformes as a whole. These studies found the problematic family Psettodidae in other parts of the diverse clade of acanthomorphs Carangaria (Li et al. 2011, Near et al. 2013, Betancur-R et al. 2013a, Campbell et al. 2013, Shi et al. 2018), while others still supported a monophyletic Pleuronectiformes (Berendzen & Dimmick 2002, Betancur-R et al. 2013b, Betancur-R & 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.

Despite widespread disagreement among studies, comprehensive taxonomic and genetic sampling has resulted in some relationships being consistent. Campbell et al. (2019) provided the only molecular phylogenetic analysis that includes all 16 flatfish families to date, Betancur-R et al. (2013b) and Betancur-R & Ortí (2014) used the largest flatfish datasets of protein-coding genes produced via the Sanger sequencing method (Sanger et al. 1977), and Harrington et al. (2016) used the only genome-wide dataset of flatfishes. All these studies converged on the same family-level topology, referred to in this study as the Betancur-Harrington-Campbell (BHC) model (Figure 1).

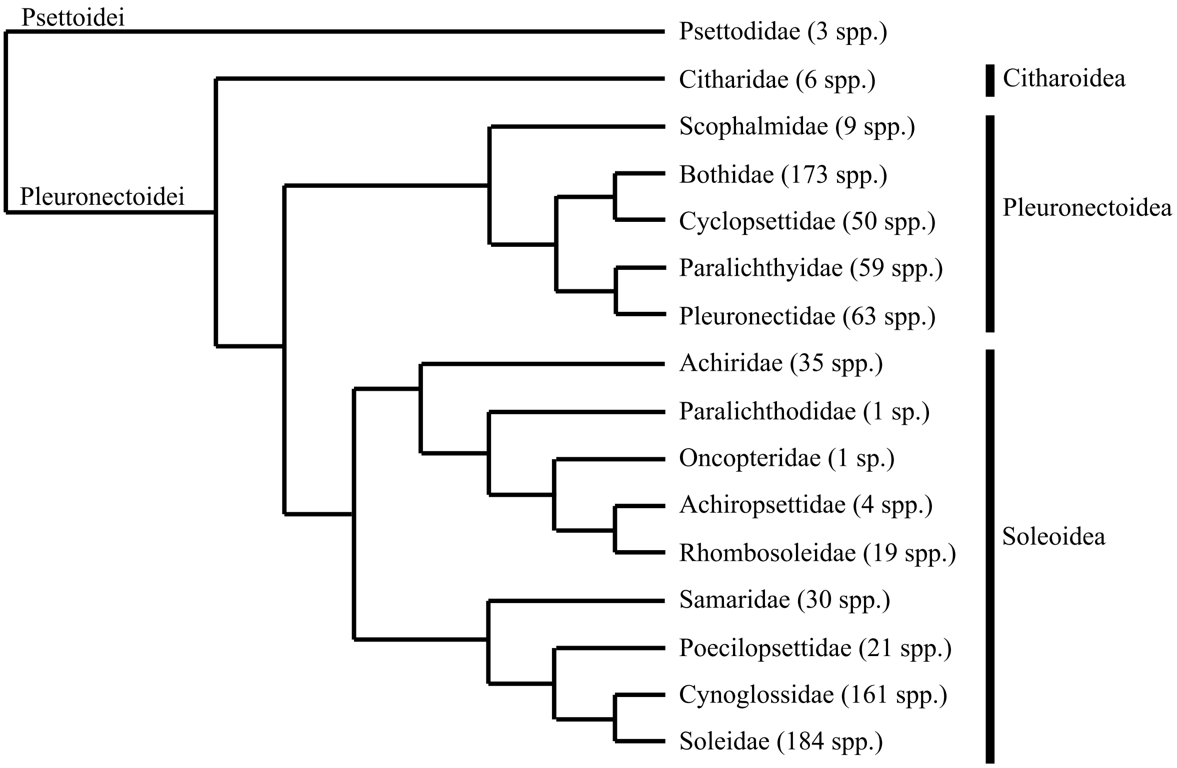

Figure 1: The BHC model of the flatfish tree based on phylogenies from Betancur-R et al. (2013b), Betancur-R & Ortí (2014), Campbell et al. (2019), and Harrington et al. (2016), with number of species in each family as of September 2020 (Fricke et al. 2020).

Taxonomic and systematic history by family

There is a rich body of literature exploring the evolution of flatfishes at deep evolutionary timescales at or beyond the family-level, but knowledge of species-level relationships is severely lacking in many parts of the flatfish tree. In order to identify what is known and where phylogenetic uncertainties remain, the current status of each family is described below. For each of the 16 flatfish families, the following is reported: (1) what the group is comprised of largely based on Fricke et al. (2020), (2) how the family was established and the evidence supporting monophyly or non-monophyly, (3) hypotheses of placement within the broader Pleuronectiformes, and (4) the extent of our knowledge on the species relationships. Tree figures are shown where there is sufficient phylogenetic evidence, and poorly resolved relationships or topologies that are conflicting between studies will be expressed as polytomies.

Psettodidae

The Psettodidae contains a single genus of spiny turbots *Psettodes* with three species, *P. belcheri*, *P. benneti*, and *P. erumei*, from the tropical Atlantic and Indo-Pacific. *Psettodes* has long been hypothesized to be sister to all other flatfishes (Pleuronectoidei) (Norman 1934). This hypothesis is supported by the adults being the least asymmetrical of all the flatfishes, and having many characters that distinguish this group from the Pleuronectoidei: spines in the pelvic fin (V I,5) and anterior part of the dorsal fin, having a maxillary with a supplementary bone, and toothed palatine (Norman 1934). A number of molecular studies also recover this relationship (Berendzen & Dimmick 2002, Li et al. 2009, Betancur-R et al. 2013b, Betancur-R & Ortí 2014, Harrington et al. 2016), but many have recovered this lineage in other parts of the Carangaria (Smith & Wheeler 2006, Li et al. 2011, Near et al. 2013, Betancur-R et al. 2013a, Campbell et al. 2013, Shi et al. 2018), dissolving the monophyletic status of the Pleuronectiformes and supporting earlier suspicions that the *Psettodes* lineage represents a second origin of the flatfish body plan (Kyle 1923, Chabanaud 1949). Phylogenetic disagreement likely stems from a large radiation early on in Carangaria evolution (Ribeiro et al. 2018), creating a period of short internal branches, followed by extremely long terminal branches. This combination produces an initial radiation where few synapomoprhies accumulate, followed by a long period where many other mutations could accumulate and potentially be masked by multiple consecuative mutations, leading to homoplasy (long branch attraction).

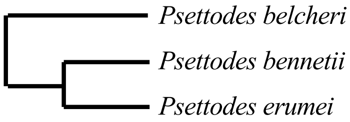

Figure 2: Psettodidae species phylogeny based on Shi et al. (2018), but poorly supported (Bootstrap Value = 62).

Citharidae

The family Citharidae consists of six species within five genera: *Brachypleura novaezeelandiae*, *Citharoides macrolepidotus*, *Citharoides orbitalis, Citharus linguatula, Paracitharus macrolepis, Lepidoblepharon ophthalmolepis*. Many taxonomists have questioned the monophyly of these six species and strong supporting evidence remains elusive (Amaoka 1972, Hensley & Ahlstrom 1984, Aboussouan 1988, Chapleau 1993, Cooper & Chapleau 1998a, Byrne et al. 2018, Shi et al. 2018). Regan (1910) had placed the genus *Brachypleura* in the Pleuronectidae subfamily Samarinae. Hoshino (2001) was able to unite the Citharidae with six characters: (1) exoccipitals forming ventral margin of foramen magnum, but zygapophyses not mutually attached; (2) teeth on epibranchial 3; (3) arterial canal perforating anterior ceratohyal; (4) first dorsal proximal radials firmly wedged into notch of blind-side lateral ethmoid; (5) anterior dorsal proximal radials tightly mutually attached; (6) blind-side posterior nostril considerably enlarged. Most molecular based phylogenies have supported the monophyly of the Citharidae (Betancur-R & Ortí 2014, Chapleau 1993, Campbell et al. 2019) with a couple exceptions (Byrne et al. 2018, Shi et al. 2018). Questionable support likely stems from its species being morphologically and genetically disparate from one another following an initial period of rapid divergence (Campbell et al. 2019). It should be noted that none of the molecular studies have included all genera. The largest taxonomic sampling was done by Campbell et al. (2019) which contains four of the five genera, excluding *Paracitharus*. The most recently described species, *Citharoides orbitalis*, has still not been sampled by any molecular phylogenies. The positioning of Citharidae within the Pleuronectiformes has not been completely resolved. Currently, there are two main hypotheses. Hypothesis 1: Citharidae is the sister clade to all other Pleuronectoidei (BHC model); Hypothesis 2: Citharidae is the sister clade to Achiridae (Azevedo et al. 2008, Byrne et al. 2018, Shi et a. 2018). Hypothesis 2 has only been recovered in studies that infer from a dataset exclusively or largely comprised of mitochondrial DNA, and these studies did not support the monophyletic status of this group.

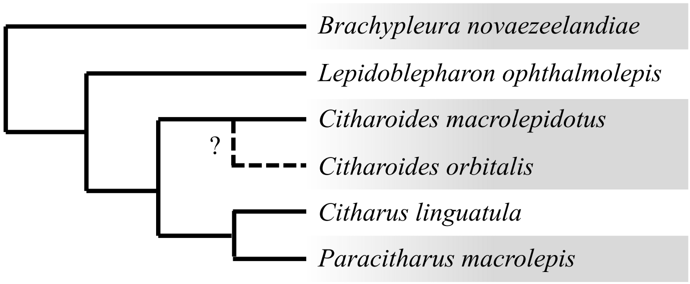

Figure 3: Citharidae species phylogeny based on Campbell et al. (2019) with placement of *Paracitharus* inferred from Byrne et al. (2018); topology also supported by Betancur-R & Ortí (2014); *Citharoides orbitalis* is not in any phylogeny.

Achiridae

Achiridae, commonly known as the American soles, is comprised of 35 dextral species within six genera: *Catathyridium* (4 spp.), *Gymnachirus* (3 spp.), *Trinectes* (10 spp.), *Apionichthys* (8 spp.), *Hypoclinemus* (1 spp.), and *Achirus* (9 spp.). The position of Achiridae within the greater Pleuronectoidei is fairly unstable, however there are a few major hypotheses. Hypothesis 1: Achiridae is sister to the Paralichthodidae-Oncopteridae-Achiropsettidae-Rhombosoleidae clade (Betancur-R & Ortí 2014, Harrington et al. 2016, Campbell et al. 2019); Hypothesis 2: Achiridae is sister to the Citharidae (Azevedo et al. 2008, Byrne et al. 2018, Shi et al. 2018); Hypothesis 3: Achiridae is sister to the Samaridae-Poecilopsettidae-Cynoglossidae-Soleidae clade (this study). In each scenario, the common ancestor between Achiridae and other groups is placed near the base of the flatfish phylogeny and more often within a sister clade to the Citharidae containing all other Pleuronectoidei. The only molecular phylogeny containing all genera of Achiridae was constructed by Byrne et al. (2018), in which the family is recovered as monophyletic along with all genera except *Trinectes* (*T. microphthalmos* and a group containing

T. maculatus, *T. paulistansus*, and *T. inscriptus* appear as two successive branches). The only other studies that use more than two genera are Azevedo et al. (2008) and Betancur-R & Ortí (2014). The latter two support the topology from Byrne et al. (2018) but do not have the taxonomic coverage to confirm or reject a paraphyletic *Trinectes* hypothesis. Azevedo et al. (2008) recovered an alternate paraphyletic *Trinectes* relationship to that from Byrne et al. (2018) and also reported a different topology overall; however, this phylogeny used fewer genes (two as opposed to nine) and taxa (four of six genera).

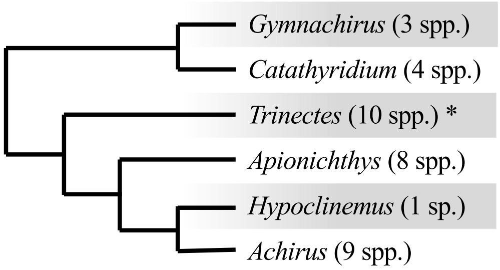

Figure 4: Achiridae genera phylogeny inferred by Campbell et al. (2019); (*) questionably monophyletic *Trinectes* is collapsed into a single branch.

Rhombosoleidae

The Rhombosoleidae is a group of dextral flounders from Australia and New Zealand and contains 19 species in 8 genera: *Ammotretis* (5 spp.), *Azygopus* (2 spp.), *Colistium* (2 spp.), *Pelotretis* (1 sp.), *Peltorhamphus* (3 spp.), *Psammodiscus* (1 sp.), *Rhombosolea* (4 spp.), and *Taratretis* (1 sp.). This group was formerly a subfamily in the Pleuronectidae (Norman 1934) and also included *Oncopterus darwinii*, now in its own family (Campbell et al. 2019). Most molecular phylogenies place the Rhombosoleidae as the sister taxon to the Antarctic Achiropsettidae, with both families forming the clade sister to Oncopteridae (Betancur-R et al. 2013b, Betancur-R & Ortí 2014, Byrne et al. 2018, Shi et al. 2018, Campbell et al. 2019). . The monophyletic status of Rhombosoleidae and the relationships between its species is complex. Guibord (2003) provides the only phylogenetic study comparing all representative species. In his analysis of 87 morphological characters he found the group to be monophyletic if *Oncopterus*, *Psammodiscus*, and *Azygopus* were excluded. *Oncopterus* was reclassified under its own separate family (Campbell et al. 2019), however the questionable placement of *Psammodiscus*, and *Azygopus* have not yet been addressed. Like *Oncopterus, Psammodiscus* was excluded from both the Achiropsettidae clade and the remaining Rhombosoleidae in Guibord (2003), however no molecular studies have included *Psammodiscus* to evaluate this hypothesis. Guibord (2003) recovered *Azygopus* as the sister taxon to the achiropsettid genus *Mancopsetta*, leading to his conclusion that *Azygopus* should be moved to the Achiropsettidae, but no molecular studies have included both *Azygopus* and species of Achiropsettidae. Further complicating the systematics in this group are the overall topologies of the most densely sampled phylogenies, which are extremely incongruent with one another (Guibord 2003, Campbell et al. 2019). In addition to *Psammodiscus*, *Taratretis* has also never been included in molecular phylogenetic analysis, and Guibord (2003) found questionable support for a monophyletic *Colistium*.

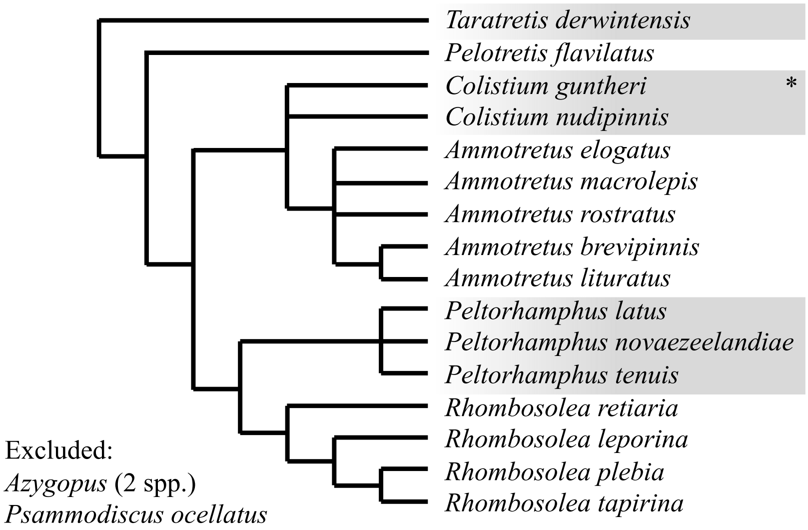

Figure 5: Rhombosoleidae species phylogeny based on Guibord (2003); (*) indicates potentially non-monophyletic.

Achiropsettidae

Achiropsettidae consists of four monotypic genera (*Achiropsetta tricholepis*, *Mancopsetta maculata*, *Neoachiropsetta milfordi*, and *Pseudomancopsetta andriashevi*) that are exclusively found in the Southern Ocean. This group was considered a part of the Bothidae by Evseenko (1985, 1996), which was assumed to be the case in Chapleau's (1993) major reassessment of the Pleuronectiformes, but the group was excluded from his analysis. Evseenko (2000) established the group as a family based on a synapomorphy in their pterygiophores following the discovery and description of *P. andriashevi* (Evseenko 1985), and placed the group as sister to the Samaridae-Achiridae-Soleidae-Cynoglossidae clade from Chapleau's (1993) phylogeny. Molecular phylogenies have in contrast shown strong support for the group being sister to Rhombosoleidae (BHC model, Shi et al. 2018, Byrne et al. 2018). This has initiated contention over whether this group should be reestablished as a subfamily within the Rhombosoleidae, some treating it as such (Byrne et al. 2018, Betancur-R et al. 2013b, Betancur-R et al. 2017), however this argument has been overshadowed by uncertainties in other parts of the pleuronectid tree. Regardless of our naming conventions for these families, if they represent a pair of sister lineages, then we can place this group in a geographic context and reasonably hypothesize that the vicariance event that separated the Rhombosoleidae (found in temperate Australia and New Zealand) and the Achiropsettidae was driven by the breakup of Australia and Antarctica roughly 30Mya. No phylogenetic studies have examined the intergeneric relationships of Achiropsettidae and *Pseudomancopsetta* has never been included in a molecular phylogenetic analysis.

Oncopteridae

The monotypic family Oncopteridae contains the dextral species *Oncopterus darwinii* Steindachnre 1874, which is known from the Southwestern Atlantic. *Oncopterus* was classified within the group now recognized as Rhombosoleidae (Norman 1934) but phylogenetic analyses have suggested that it is sufficiently distinct to be in its own family (Guibord 2003), especially if the Achiropsettidae is recognized as a family since *Oncopterus* is recovered as the sister lineage to the Achiropsettidae-Rhombosoleidae clade in several studies (Betancur-R & Ortí 2014, Byrne et al. 2018, Campbell et al. 2019). Campbell et al. (2019) formally classified the species within its own family and identified seven defining characters that were first observed by Norman (1934) and Chapleau (1993): (1) eyes on the right side; (2) large foramen on branchial septum between lower pharyngeals and urohyal; (3) origin of dorsal fin anterior to the eyes above blind-side nostrils; (4) first dorsal-fin ray specialized, enlarged, hard, curved, and movable, and connects with first strongly developed basal bone of the fin, contained in a deep groove on blind-side head; (5) pelvic fins asymmetric and separated by anal fin, ocular-side pelvic fin located much more anterior than that of the blind-side; (6) six pelvic-fin rays; (7) lateral line with distinctive semi-circular curve above the pectoral fin and several transverse supratemporal accessory branches from main lateral line to dorsal edge of body. While Campbell et al. (2019) did not register their new classification to ZooBank to be in accordance with the International Code of Zoological Nomenclature (ICZN) Article 8.5, the subfamily Oncopterinae, which is considered a family-group in the ICZN, has already been proposed (Jordan & Goss 1889), and therefore Oncopteridae is recognized as a valid family in Catalogue of Fishes (van der Laan & Fricke 2020).

Paralichthodidae

The dextral *Paralichthodes algoensis* is only known from South Africa and is the only species in the family Paralichthodidae. Placement of *Paralichthodes* within the Pleuronectiformes is still uncertain due to exclusion in most phylogenetic studies. Regan (1910) had placed the species in the Samaridae (which was at the time classified as a subfamily within the Pleuronectidae). A decade later, Regan (1920) revised this previous work, in which he placed the species within its own family, but another revision returned the species to the Plauronectidae, but this time within its own subfamily Paralichthodinae (Regan 1929). *Paralichthodes* remained within its own subfamily in the classification used by Norman (1934) and Hubbs (1945) but was once again considered a member of Samarinae in Nelson (1984). Chapleau's (1993) phylogeny of the Pleuronectiformes did not include *Paralichthodes* but the species was later added to the tree as its own family by Cooper & Chapleau (1998b), placed sister to the clade containing the Rhombosoleidae, Poecilopsettidae, Samaridae, Achiridae, Soleidae, and Cynoglossidae. This placement was largely based *Paralichthodes* lacking synapomorphies that were present in other groups. The first and only molecular study to include *Paralichthodes* places it as sister to the clade containing Oncopteridae, Achiropsettidae, and Rhombosoleidae (Campbell et al. 2019).

Samaridae

The crested flounders in the Samaridae are represented by 30 dextral species in four genera: *Plagiopsetta* (4 spp.), *Samaretta* (1 sp.), *Samaris* (5 spp.), *Samariscus* (20 spp.). They are a well-supported monophyletic group (Chapleau & Keast 1988, Alfonso 2001, Berendzen & Dimmick 2002, Azevedo et al. 2008, Betancur-R & Ortí 2014, Byrne et al. 2018, Campbell et al. 2019) that can be distinguished from other flatfishes by [1] their lack of a blind-side pectoral fin, [2] strait lateral line, [3] small mouth, [4] short gill rakers, [5] asymmetrical pelvic fins, [6] small scales, and [7] minute blind-side nostrils (Alfonso 2001). The family was originally a subfamily in the Pleuronectidae (Regan 1910) and included two citharid genera *Brachypleura* and *Lepidoblepharon* that were removed by Hubbs (1945). Chapleau & Keast (1988) elevated the group to a family and placed the Samaridae as sister to the Achiridae-Cynoglossidae-Soleidae clade. The relationship between Samaridae and Achiridae is unstable, but the Samaridae consistently recovered near the Soleidae-Cynoglossidae clade, most commonly preceded by Poecilopsettidae (BHC model, Byrne et al. 2018). Alfonso (2001) provided the most comprehensive phylogenetic study on the group and concluded that the three genera known at the time were all monophyletic and provided synapomorphies for each. *Plagiopsetta* has (1) an unpigmented snout, (2) 7-9 pectoral rays, and (3) a blind-side with some degree of pigmentation. *Samaris* has (1) prolonged dorsal rays, (2) prolonged ocular pelvic rays, and (3) simple caudal rays. *Samariscus* is the only Samaridae with 5 abdominal vertebrae baring paired parapophyses (rather than 6 or 7). The recently discovered *Samaretta perexilis* is only known from deep-water on Southeastern Pacific seamounts and is characterized by unremarkable or not elongated anterior dorsal fin rays, four pectoral fin rays, six parapophyses, reduced lateral line canals, a very slender body, large head and eyes, and minute scales (Voronina & Suzumoto 2017). The relationships between Samaridae genera and species are poorly resolved. Alfonso (2001) show *Samaris* and *Samariscus* as sister, whereas Betancur-R & Ortí (2014) and Ji et al. (2016) recover *Plagiopsetta* and *Samariscus* as sister, and in the Campbell et al. (2019) phylogeny *Plagiopsetta* and *Samariscus* are sister. Results from Byrne et al. (2018) corroborated the finding of Campbell et al. (2019), but also detected a non-monophyletic *Samariscus*, with *S. triocellatus* sister to all other Samaridae. It is unclear if this is indicative of two lineages of *Samariscus* or is an artifact of genetic bias. *Samaretta perexilis* has not yet been included in any phylogenetic analyses.

Alfonso (2001) suggested one potential monophyletic group within *Samariscus* composed of *S corallinus*, *S. nielseni*, and *S. triocellatus,* as they are united by several unique characters: (1) first few dorsal fin rays free of the membrane; (2) ocelli present; (3) snout longer than the eye; (4) blind-side tissue flap at the base of the first dorsal fin ray.

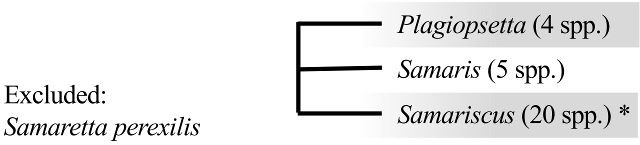

Figure 6: Samaridae genera trichotomy jointly inferred from Guibord (2003), Betancur-R & Ortí (2014), and Campbell et al. (2019); (*) indicates potentially non-monophyletic; *Samariscus* is non-monophyletic in Byrne et al. (2018).

Poecilopsettidae

The Poecilopsettidae is a group of 21 species of dextral flounders in three genera: *Marleyella* (2 spp.), *Nematops* (4 spp.), *Poecilopsetta* (15 spp.). They are found primarily in the tropical and subtropical Indian and Pacific oceans with the exception of two species, *P. beanii* and *P. inermis*, from the Western Atlantic. This family is characterized by having large dextral eyes, the absence of the lateral line on the blind-side, a wide proximal pterygiophore zone without muscles, and the absence of orbital and preorbital bones (Guibord 2003). Poecilopsettinae was originally a subfamily within the Pleuronectidae (Norman 1984) but was elevated to family status by Chapleau (1993). Both the phylogenetic position of the Poecilopsettidae and the relationships between its species are poorly resolved. A comprehensive phylogenetic analysis of the relationships within Poecilopsettidae has never been done, but in a taxonomic revision of the family, Guibord (2003) found evidence for the two genera *Marleyella* and *Nematops* to be monophyletic but suspected a non-monophyletic *Poecilopsetta* due to the absence of synapomorphies. The sexually dimorphic *Marleyella* is characterized by the presence of rostral spines in both sexes (stronger in males), elongated first few dorsal and pelvic fin rays (longer in males), and blue branchiostegal membranes in both sexes (Guibord 2003). *Nematops* is characterized by having an eyed-side nasal bone, having three proximal radials of the anal fin found between the first enlarged pterygiophore of the anal fin and the haemal spine of the first caudal vertebra, and many but not all specimens having a tentacle on both eyes or just the ventral eye (Guibord 2003). The family Poecilopsettidae has appeared in many different places in the broader Pleuronectiformes tree, the most common being the sister group to the clade containing the Cynoglossidae and Samaridae (BHC model, Byrne et al. 2018).

Soleidae

The true soles of the family Soleidae are a group of almost invariably dextral flatfishes inhabiting old-world marine habitats (Hubbs 1945). This diverse family contains 184 species in 30 genera: *Achiroides* (2 spp.), *Aesopia* (1 sp.), *Aseraggodes* (54 spp.), *Austroglossus* (2 spp.), *Barbourichthys* (1 sp.), *Barnardichthys* (1 sp.), *Bathysolea* (4 spp.), *Brachirus* (20 spp.), *Buglossidium* (1 sp.), *Dagetichthys* (6 spp.), *Dexillus* (1 sp.), *Dicologlossa* (1 sp.), *Heteromycteris* (6 spp.), *Leptachirus* (8 spp.), *Liachirus* (2 spp.), *Microchirus* (7 spp.), *Monochirus* (3 spp.), *Paradicula* (1 sp.), *Pardachirus* (7 spp.), *Pegusa* (6 spp.), *Phyllichthys* (3 spp.), *Rendahlia* (1 sp.), *Rhinosolea* (1 sp.), *Solea* (9 spp.), *Soleichthys* (10 spp.), *Synapturichthys* (1 sp.), *Synclidopus* (2 spp.), *Typhlachirus* (3 spp.), *Vanstraelenia* (1 sp.), and *Zebrias* (19 spp.). Soleidae was originally one of two families within the Heterosomata (the other being Pleuronectidae) and was dedicated for all flatfishes commonly referred to as “soles” (Jordan & Evermann 1898). It included the subfamilies Soleinae, Cynoglossinae, and Achirinae. Hubbs (1945) elevated Cynoglossinae to family level and Chapleau & Keast (1988) elevated Achirinae to family level, restricting Soleidae to only include species in what was previously the subfamily Soleinae. The Soleidae (*sensu* Chapleau & Keast 1988) is well established as the sister clade to Cynoglossidae (BHC model, Chapleau 1993, Byrne et al. 2018). Two molecular studies found incongruent results. Berendzen & Dimmick (2002) failed to recover a monophyletic Soleidae and Shi et al. (2018) found Soleidae to be sister to the Samaridae. The Cynoglossidae-Soleidae clade is supported by seven synapomorphies: (1) portion of blind-side dentary anterior to anguloarticular is convex and shorter and deeper than eyed-side bone; (2) totally concealed margin of preopercle; (3) deep fimbriation pattern on subopercle; (4) reduced (if present) eyed-side mesopterygoid; (5) blind-side ethmoid only bone forming blind-side margin of upper eye orbit; (6) first proximal radial of dorsal fin with long anterior process; (7) hypurals 1 to 4 fused to first preural centrum (except in *Symphurus australis* in sample of three *Symphurus spp.*) (Chapleau & Keast 1988), and in addition to being dextral, the Soleidae are morphologically distinct from the Cynoglossidae by six synapomorphies: (1) vomer protruding ventrally; (2) metapterygoid with high perpendicular lamina along its posterodorsal margin; (3) ascending process of blind-side premaxilla is always directed toward eyed-side and extends in front of rostral cartilage and opposing premaxilla; (4) maxillary spike of eyed-side maxilla covering rostral cartilage dorsally; (5) blind-side lateral ethmoid with posteroventral process attached to sphenotic; (6) basioccipital with two distinct ventral processes for articulation of branchial arches (except in *Pardachirus*) (Chapleau & Keast 1988). A number of molecular analyses have examined this group including several full mitochondrial genomes (Wang et al. 2013, Shi et al. 2014, 2016, Gong et al. 2015, 2016), and many have attempted to elucidate the intrafamilial systematics of the Soleidae, but most lack the taxonomic breadth required to infer phylogenetic relationships across the entire family (Tinti et al. 2000, Borsa & Quignard 2001, Infante et al. 2004, Pardo et al. 2005, Gong et al. 2018). Byrne et al. (2018) provides a comprehensive sampling of 44 species and 17 genera, but their results are difficult to interpret due to 5 of the 13 polytypic genera being recovered as polyphyletic (*Aseraggodes, Pardachirus, Pegusa, Solea, Zebrias*). Other studies agree with the overall topology but do not find abundant polytomies albeit with very limited sampling (Infante et al, 2004, Betancur-R & Ortí 2014, Campbell et al. 2019), so it is unclear to what extent the results from Byrne et al. (2018) represent true non-monophyletic genera or if there were artifacts from incomplete lineage sorting (ILS) and/or a limited genetic dataset of nine markers. Furthermore, 13 genera have yet to be genetically sampled: *Achiroides*, *Barbourichthys*, *Barnardichthys*, *Dexillus*, *Leptachirus*, *Liachirus*, *Paradicula*, *Phyllichthys*, *Rendahlia*, *Rhinosolea*, *Synclidopus*, *Typhlachirus*, and *Vanstraelenia*. The *Soleidae* are in need of phylogenetic revision using dense sampling of all genera, especially from those that are suspected to be non-monophyletic, and using larger character datasets.

Cynoglossidae

The tonguesoles in the Cynoglossidae are a large group of morphologically distinct flatfishes that occur primarily in shallow and estuarine habitats, but some occur in rivers and in the deep sea. The family consists of 161 sinistral species within three genera *Cynoglossus* (74 spp.), *Paraplagusia* (6 spp.), and *Symphurus* (81 spp.). The Cynoglossinae was a subfamily of Soleidae (Jordan & Evermann 1898) and was elevated to its current family status by Hubbs (1945). Chapleau & Keast (1988) used a large osteological dataset to identify 22 characters to differentiate the Cynoglossidae from their well-established sister group Soleidae, most notably being entirely sinistral, having a rostral hook extending anterodorsally over the mouth, lacking pectoral fins, and lacking pelvic fin on the ocular-side. Chapleau & Keast (1988) also analyzed the relationships between the three genera, the topology of which is replicated using molecular datasets (Betancur-R & Ortí 2014, Shi et al. 2018, Campbell et al. 2019). *Cynoglossus* and *Paraplagusia* are sister taxa in the subfamily Cynoglossinae, united with six characteristics.

Paraplagusia is monophyletic, united by five synapomorphies, and *Cynoglossus* is non-monophyletic with several lineages more closely related to *Paraplagusia* than to other congeneric members. *Symphurus* is monophyletic within its own subfamily Symphurinae.

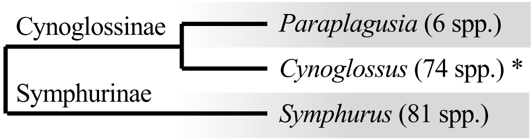

Figure 7: Cynoglossidae genera phylogeny based on Chapleau & Keast (1988). (*) indicates potentially non-monophyletic; *Cynoglossus* is not monophyletic in Chapleau & Keast (1988) and Byrne et al. (2018).

Scophthalmidae

Scophthalmidae is comprised of nine sinistral species within three genera (*Lepidorhombus*, *Scophthalmus*, and *Zeugopterus*) from European waters including the Mediterranean Sea, Baltic Sea, Black Sea. The monophyletic status of the family is well supported by many morphological and molecular phylogenies (Hensley & Ahlstrom 1984, Chapleau 1993, Hoshino & Amaoka 1998, Berendzen & Dimmick 2002, Chanet 2003, Azevedo et al. 2008, Betancur-R & Ortí 2014, Byrne et al. 2018, Campbell et al. 2019). It used to be a subfamily within the Bothidae (Norman 1934), but has been its own family since Hubbs (1945). Chanet (2003) did a comprehensive morphological analysis on the family and identified five synapomorphies: (1) an anterior extension of the two pelvic fins to the isthmus (Chapleau 1993), (2) a supraoccipital with an anterior process (often crenulated, forming a bridge with the dorsal margin of the right frontal (Chapleau 1993), (3) lateral expansions (slightly asymmetric) on both pelvic bones (Chanet 1998), (4) asymmetrical transverse apophyses on the caudal vertebrae, (5) first neural spine bent at contact with the dorsal margin of the cranium. The position of Scophthalmidae within the broader Pleuronectoidei remains enigmatic. In the Regan-Norman model it is a sister group to the citharid subfamily Citharinae (Hensley & Ahlstrom 1984) within the “bothoid” group (*sensu* Hensley & Ahlstrom 1984). This placement was based on a caudal complex structure definitive of the “bothoid” group, which is now recognized as polyphyletic. One of the leading hypotheses is that Scophthalmidae is the sister group to the Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae clade, first proposed by Chapleau (1993). This relationship appears in a number of molecular phylogenies (BHC model) and Campbell et al. (2019) refers to this unified group as the superfamily Pleuronectoidea. Another relationship that has been reported is Scophthalmidae being the sister group to the Rhombosolidae based on datasets of primarily mitochondrial genes (Byrne et al. 2018, Shi et al. 2018). There is less ambiguity concerning the interrelationships within Scophthalmidae. Chanet (2003) identified synapomorphies of all three genera (2 for *Scophthalmus*, 2 for *Lepidorhombus*, and 3 for *Zeugopterus*), five supporting a sister-relationship between *Lepidorhombus* and *Scophthalmus*, three supporting a sister-relationship between *Zeugopterus punctatus* and *Z. regius*, and four supporting a sister-relationship between *Scophthalmus rhombus* and *S. aquosus*. The most comprehensive molecular dataset also supports these relationships (Byrne et al. 2018) with exception of one rogue *S. aquosus* sample positioned far outside of its family, however, other molecular studies with *S. aquosus* reconfirm its intrafamilial placement (Azevedo et al. 2008, Betancur-R & Ortí (2014). Azevedo et al. (2008) found a different arrangement within *Scophthalmus*, placing *S. aquosus* as sister to *S. maximus* rather than *S. rhombus*, but this has not been recovered in any other studies nor is it supported by morphological characters. *Scophthalmus maeoticus* was considered a subspecies of *S. maximus* in Chanet’s (2003) assessment, but has since been considered valid in a number of checklists (Parin et al. 2014, Çiçek et al. 2015, Artüz and Fricke 2019) based on morphology. Molecular data hints toward *S.*

maeoticus not being a genetically distinct species from *S. maximus* (Byrne et al. 2018). *Z. regius* has yet to be genetically sequenced.

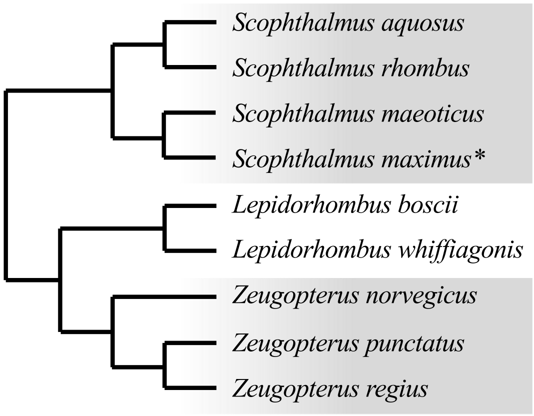

Figure 8: Scophthalmidae species phylogeny based on Chanet (2003) with position of *S. maeoticus* inferred from Byrne et al. (2018); (*) *S. maximus* is potentially non-monophyletic, with the morphologically distinct *S. maeoticus* potentially being a subspecies of *S. maximus* based on Byrne et al. (2018).

Pleuronectidae

The Pleuronectidae (*sensu* Chapleau & Keast 1988) contains 63 species in 24 families. These fishes inhabit temperate and polar waters in the northern hemisphere and many are valued by commercial fisheries such as the Pacific halibut (*Hippoglossus stenolepis*). Although they are commonly referred to as “right-eyed flounders” this group does not contain all dextral species of Pleuronectiformes. Additionally, this group contains a small number of species for which both dextral and sinistral individuals have been found, most notably the starry flounder (*Platichthys stellatus*). This discontinuity in naming stems from a complex history with many previous iterations of the family that were largely disregarded as a cladistically relevant taxon due its treatment as a “trash bin” taxonomic group (Chapleau 1993). Pleuronectidae had originally been designated for all flatfish species, with the current definition restricted to the subfamily Pleuronectinae, along with the subfamilies Hippoglossinae, Platessinae, Soleinae, Cynoglossinae, Samarinae, and Oncopterinae (Cuvier 1816, Jordan & Goss 1889). The flatfishes were later redefined as the taxon Heterosomata, containing two families: Pleuronectidae (Hippoglossinae, Pleuronectinae, and Platessinae) and Soleidae (Soleinae and Cynoglossinae) that were distinguished by the preopercle margin, eye size and position, and prominence of teeth (Jordan & Evermann 1898). Reference to the Pleuronectidae as the “right-eyed flounders” originates from Regan (1910), who restricted the Pleuronectidae to only contain dextral flounders (Pleuronectinae, Rhombosoleinae, Samarinae) with five uniting characteristics in total identified. This family of dextral flatfishes was further refined to include the subfamilies Pleuronectinae, Rhombosoleinae, Samarinae, Poecilopsettinae, and Paralichthodinae and united by 14 characteristics (Regan 1920, 1929, Jordan 1923, Norman 1934), but many of these defining characters were not unique to this group (Hensley & Ahlstrom 1984). Hubbs (1945) pulled *Brachypleura* and *Lepidoblepharon* out of the Pleuronectidae and reclassified these genera as being within the Citharidae, further questioning characters that distinguish the Pleuonectidae from other flatfishes. Nelson (1984) reduced the number of defining characteristics of Pleuronectidae to two of the original traits from Regan (1910): eyes almost always dextral and no oil globule in yolk of egg. The same year, Hensley & Ahlstrom (1984) questioned the monophyletic status of the Pleuronectidae (*sensu* Norman 1934) on the basis of many traits being plesiomorphic and asserting that although eyedness in flatfishes is easily identifiable, it may not be the most evolutionarily informative trait. In the first major phylogenetic assessment of the flatfish order, now called Pleuronectiformes, Chapleau (1993) validated the polyphyletic status of the Pleuronectidae (*sensu* Norman 1934), and formally established the contemporary definition of Pleuronectidae, first used in Chapleau & Keast (1988). All other subfamilies in Pleuronectidae (*sensu* Norman 1934) were also elevated to their current families (Chapleau 1993, Cooper & Chapleau 1998b). A consequence of this major breakup of the Pleuronectidae (*sensu* Norman 1934) was that characters previously used to define Pleuronectidae were no longer valid and could not be synapomorphies for this group. Cooper & Chapleau (1998a) completed the first comprehensive phylogenetic analysis on the Pleuronectidae (*sensu* Chapleau & Keast 1988), examining 106 morphological characters and 53 of the 59 species recognized at the time and identified ten synapomorphies for the family: (1) ocular-side frontal articulated with mesethmoid; (2) Ocular-side preorbital sensory canal absent except in *Reinhardtius hippoglossoides* and *Acanthopsetta nadeshnyi*; (3) ventral margin of metapterygoid flattened except in *Atheresthes stomias*.; (4) first and second basibranchials loosely joined by cartilage except in seven phylogenetically distant species; (5) second and third basibranchials loosely joined by cartilage except in two of the previous species *Myzopsetta punctatissima* and *Psettichthys melanostictus*; (6) posteriormost abdominal vertebrae lack haemapophysis except in *Atheresthes stomias*, *Eopsetta grigorjewi*, and some *Microstomus* spp.; (7) accessory process on caudal vertebrae absent except in both *Hippoglossus* spp. and in some *Microstomus* and *Pleuronichthys* spp.; (8) ocular-side infraorbital bones present except in *M. bathybius*; (9) oil globules in egg absent except in three *Pleuronichthys* spp.; (10) olfactory laminae are parallel without a central rachis except in both *Atheresthes* spp. Cooper & Chapleau (1998a) also examined the interrelationships of Pleuronectidae (*sensu* Chapleau & Keast 1988), of which previous studies are sparse. Norman (1934) defined two tribes Hippoglossini and Pleuronectini based on jaw morphology and Sakamoto (1984) was the first to review these interrelationships, but these models were not widely accepted. Cooper & Chapleau (1998a) revised the family classification to include five subfamilies Hippoglossinae, Eopsettinae, Lyopsettinae, Hippoglossoidinae, and Pleuronectinae, all with defining traits. Since molecular tools have become more widely available, a number of studies have reexamined the interfamilial relationships using genetic data (Kartavtsev et al. 2008a, 2008b, 2016, Roje 2010, Ji et al. 2016, Vinnikov et al. 2018), but none support the morphologically inferred relationships from Cooper & Chapleau (1998a). Of these, Vinnikov et al. (2018) provides the most comprehensive study to-date, sampling from 60 of 63 recognized species and using sequences from seven genes. Their study established the five subfamilies currently recognized – Atheresthinae, Pleuronichthyinae, Microstominae, Hippoglossinae, and Pleuronectinae – the latter two containing a different assemblage of species from their definitions proposed by Cooper & Chapleau (1998a). Atheresthinae contains two species in the genus *Atheresthes* and is sister to all other Pleuronectidae, and Pleuronichyinae contains eight species in the genus *Pleuronichthys* and is sister to the clade containing the remaining three subfamilies.

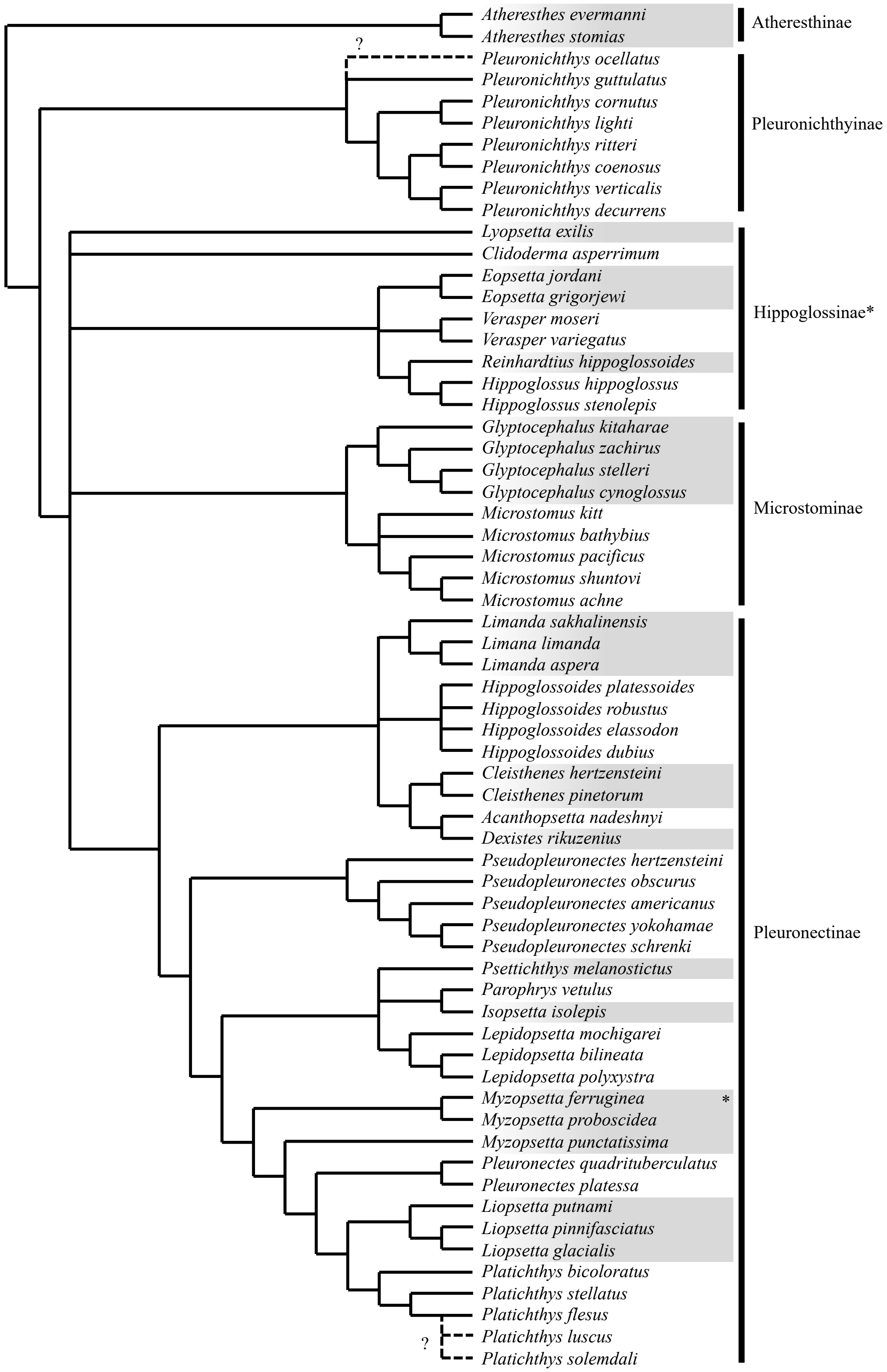

Figure 9: Conceptual phylogeny of the Pleuronectidae species based on consensus between Rohe (2010) and Vinnikov et al. (2018) with unresolved relationships collapsed into polytomies. The positions of *Pleuronichthys oscellatus*, *Platichthys luscus*, and *P. solemdali* are unknown. '*' indicates non-monophyletic. "Hippoglossinae" = Hippoglossinae (*sensu* Vinnikov et al. 2018) excluding *Clidoderma* and *Lyopsetta*.

Paralichthyidae

The Paralichthyidae (*sensu* Betancur-R et al. 2017) is comprised of 59 species in ten genera: *Ancylopsetta* (6 spp.), *Cephalopsetta* (1 sp.), *Gastropsetta* (1 sp.), *Hippoglossina* (6 spp.), *Paralichthys* (19 spp.), *Pseudorhombus* (25 spp.), *Tarphops* (2 spp.), *Tephrinectes* (1 sp.), *Thysanopsetta* (1 sp.), and *Xystreurys* (2 spp.). While this taxonomic group is invalid according to the ICZN, the name Paralichthyidae is still recognized with inclusion of four other genera in the Cyclopsettidae (Fricke et al. 2020) and there is strong molecular and morphological evidence supporting Paralichthyidae (*sensu* Betancur-R et al. 2017) and Cyclopsettidae being distinct family-level groups (Betancur-R et al. 2017). The historical classification of Paralichthyidae that included species in the Cyclopsettidae was originally a subfamily within the Bothidae (Norman 1934). This Paralichthyinae was distinguished from other Bothidae by having (nearly) symmetrical pelvic fins and lacking transverse apophyses on the vertebrae (Norman 1934). Amaoka (1969) removed the group from Bothidae and elevated it to its family status. In response to the many molecular phylogenies supporting two different clades of Paralichyidae (Berendzen & Dimmick 2002, Betancur-R & Ortí 2014, Harrington et al. 2016, Betancur-R et al. 2017, Byrne et al. 2018, Shi et al. 2018), Campbell et al. (2019) formally removed Cyclopsettidae, however, no morphological synapomorphies have been identified for the remaining Paralichthyidae (*sensu* Betancur 2017) and several molecular studies have failed to support its monophyletic status (Berendzen & Dimmick 2002, Pardo et al. 2005, Azevedo et al. 2008), instead showing it to be paraphyletic with the Pleuronectidae being nested within it. The relationship between Paralichthyidae and Pleuronectidae is still not well established, however, the group containing both families consistently resolves as sister to the Cyclopsettidae-Bothidae clade in molecular phylogenies (Berendzen & Dimmick 2002, Betancur-R & Ortí 2014, Harrington et al. 2016, Byrne et al. 2018, Shi et al. 2018, this study). The inter-species relationships of Paralichthyidae are extremely enigmatic. Several molecular phylogenetic studies have included at least three genera in their datasets (Berendzen & Dimmick 2002, Azevedo et al. 2008, Betancur-R & Ortí 2014, Byrne et al. 2018, Campbell et al. 2019), however, topologies vary widely and there is evidence for several non-monophyletic genera: *Paralichthys* and *Pseudorhombus* (Byrne et al. 2018). Moreover, no molecular phylogenies have included *Cephalopsetta* or *Thysanopsetta*. *Cephalopsetta*, *Pseudorhombus*, and *Tarphops* have been suspected to form a monophyletic clade united by the epural fused to the fifth hypural, a caudal ray count of more than 17, no splinter ray on the most ventral caudal fin ray, and urogenital papillae on the ocular side (Amaoka 1969, Hensley & Ahlstrom 1984, Munroe 2015) that is dubbed the "Pseudorhombus" group by Amaoka (1969). Two species, *Tephrinectes sinensis* and *Thysanopsetta naresi*, were suspected to not be part of the Paralichtyidae (*sensu* Amaoka 1969) based on morphology (Hensley & Ahlstrom 1984, Hoshino and Amaoka 1998, Hoshino 2001), however, the molecular phylogeny by Byrne et al. (2018) supports at least *T. sinensis* being within Paralichthyidae (*sensu* Betancur-R et al. 2017), while *T. naresi* remains to be genetically sampled.

Cyclopsettidae

Cyclopsettidae (*sensu* Betancur-R et al. 2017) is not recognized as a valid family by Eschmeyer's Catalogue of Fishes (van der Laan & Fricke 2020) 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 & 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 family contains 45 species within four genera, *Cyclopsetta* Gill 1899 (4 spp.), *Syacium* Ranzani 1842 (8 spp.), *Etropus* Jordan & Gilbert 1882 (9 spp.), and *Citharichthys* Bleeker 1862 (24 spp.), that are currently classified in the Paralichthyidae, but are more closely related to the Bothidae than to the Pleuronectidae and other Paralichthyidae. This group's position and monophyletic status are recovered in nearly all molecular phylogenies (Berendzen & Dimmick 2002, Pardo et al. 2005, Betancur-R et al. 2013b, Betancur-R & Ortí 2014, Harrington et al. 2016, Shi et al. 2018, Byrne et al. 2018, Campbell et al. 2019) with the exception of Azevedo et al. (2008) which fails to resolve the monophyly of Cyclopsettidae and also does not support the sister relationship of the Cyclopsettidae-Bothidae and Paralichthyidae-Pleuronectidae clades. Hensley & Ahlstrom (1984) pointed out that the Cyclopsettidae exhibit a unique arrangement of caudal fin rays, a potential synapomorphy for the group. Campbell et al. (2019) identified two characters described in Hensley & Ahlstrom (1984) that unite the Cyclopsettidae-Bothidae clade (absence of the first neural spine, and presence of transverse apophyses on the vertebrae) further supporting this sister relationship. The intergeneric relationships are as such: *Cyclopsetta* and *Syacium* form one monophyletic clade as sister groups to one another; *Citharichthys* and *Etropus* form another monophyletic clade but may not be reciprocally monophyletic to one another (Betancur-R & Ortí 2014, Byrne et al. 2018). Hensley & Ahlstrom (1984) were able to distinguish the *Cyclopsetta-Syacium* and *Etropus- Citharichthys* clades using larval characteristics. Khidir et al. (2005) identified an additional six synapomorphies for the *Cyclopsetta-Syacium* clade, ten for *Cyclopsetta*, and five for *Syacium*. Molecular studies containing the genera *Etropus* and/or

Citharichthys are taxonomically sparse, so further investigation is required to validate or reject the monophyletic status of these genera.

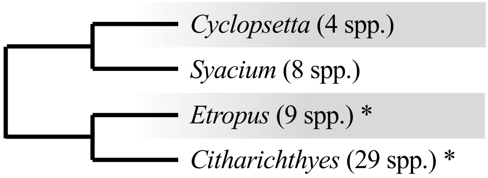

Figure 10: Cyclopsettidae genera phylogeny based on Byrne et al. (2018); (*) *Etropus* and *Citharichthys* are not reciprocally monophyletic in Azevedo et al. (2008), Betancur-R & Ortí (2014), and Campbell et al. (2019).

Bothidae

Bothidae contains 173 sinistral species in 20 genera: *Arnoglossus* (35 spp.), *Asterorhombus* (4 spp.), *Bothus* (18 spp.), *Chascanopsetta* (10 spp.), *Crossorhombus* (5 spp.), *Engyophrys* (2 spp.), *Engyprosopon* (34 spp.), *Grammatobothus* (3 spp.), *Japonolaeops* (1 sp.), *Kamoharaia* (1 sp.), *Laeops* (13 spp.), *Lophonectes* (2 spp.), *Monolene* (11 spp.), *Neolaeops* (1 sp.), *Parabothus* (10 spp.), *Perissias* (1 sp.), *Psettina* (10 spp.), *Taeniopsetta* (2 spp.), *Tosarhombus* (6 spp.), *Trichopsetta* (4 spp.). Historically this name was used in contrast to the Pleuronectidae (*sensu* Norman 1934) to represent all sinistral flounders, including what are now the Paralichthyidae, Scophthalmidae, and Achiropsettidae (Norman 1934), the former two originally distinguished as subfamilies. Although these families were extracted from the Bothidae (Scophthalmidae in Hubbs 1945, Paralichthyidae in Amaoka 1969, and Achiropsettidae in Evseenko 2000), the common name of "left-eyed flounders" has persisted with the family name. The Bothidae (*sensu* Evseenko 2000) are consistently monophyletic and are found within a clade shared with Pleuronectidae, Paralichthyidae, and Cyclopsettidae (BHC model, Cooper & Chapleau 1998b, Shi et al. 2018, Byrne et al. 2018). Berendzen & Dimmick (2002) and Azevedo et al. (2008) confirm monophyly and its sister relationship with the Cyclopsettidae (also observed in all molecular phylogenies) but report this group being phylogenetically distant from the Pleuronectidae and other Paralichthyidae, however, the genetic dataset used by these studies was small in comparison to many others. Chapleau (1993) and Hensley & Ahlstrom (1984) identified eight distinctive characters for the Bothinae (*sensu* Amaoka 1969, which at the time included what is now in the Achiropsettidae): in adults, (1) loss of the preorbital on the blind-side, (2) presence of myorhabdoi, and (3) asymmetrical states of pelvic-fin morphology; in larvae, (1) large size at metamorphosis, (2) eye migration below the dorsal fin, (3) dorsal fin origin anterior to eyes, (4) elongated second dorsal fin ray before completion of notochord flexion, and (5) lack of preopercular spines. The Achiropsettidae are further distinguished by their pterygiophores (Evseenko 2000). The relationships between Bothidae (*sensu* Evseenko 2000) species are largely unknown. Systematic studies since Amaoka's (1969) work are severely lacking, and even he did not include all genera in his assessment. Amaoka (1969) identified two suborders: Taeniopsettinae containing *Taeniopsetta*, *Engyophrys*, *Trichopsetta*, and *Perissias*, and Bothinae, contain all other genera. The most comprehensive morphological dataset, based on larval characters, shows potential support for the existence of Taeniopsettinae, but the remaining Bothinae are largely unrelated to one another and exhibit a large polytomy at the base of the tree (Amaoka et al. 1981, Fukui 1997). Molecular-based phylogenetic inference has also resulted in ambiguous species relationships with few species being represented and little overlap in taxa used among studies (Berendzen & Dimmick 2002, Azevedo et al. 2008, Betancur-R & Ortí 2014, Li et al. 2015, Shi et al. 2018, Campbell et al. 2019). Byrne et al. (2018) inferred from the largest taxonomic coverage with 47 species over 15 genera, but even this phylogeny was unable to elucidate many of the relationships, because many genera were recovered as non-monophyletic (7/12 polytypic genera, including *Arnoglossus, Crossorhombus, Psettina, Engyprosopon,* *Laeops, Chascanopsetta, Bothus*). Morphological examinations also suspected the polyphyletic status of *Arnoglossus* and *Engyprosopon* (Hensley 1986, Fukui 1997). *Perissias* and *Tosarhombus* have yet to be genetically sampled.

Chapter 1: Exon-capture dataset highlights genome-wide phylogenetic uncertainty in Pleuronectoidei

Contributions:

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Abstract

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 apparent bursts of evolution in short time periods early in the Pleuronectiformes tree and an abundance of studies that incompletely and inconsistently sample taxa and genetic markers. We present a phylogeny 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). Trees resulting from concatenation and species-tree approaches, and from a total evidence dataset and a filtered set of loci screened based on clocklikeness, are remarkably consistent with other recent molecular phylogenies, and show strong monophyletic support for all families included except Pleuronectidae. Our analysis failed to provide further resolution on some interfamilial relationships early on in the flatfish phylogeny and among some of the Pleuronectidae subfamilies; however, we resolve a novel placement for the family Rhombosoleidae as sister to a clade containing Cyclopsettidae, Bothidae, Paralichthyidae, and Pleuronectidae. 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, flatfishes, flounders, incomplete-lineage-sorting, target-enrichment

Introduction

Reaching consensus on an evolutionary hypothesis can be challenging when different phylogenetic studies result in different conclusions. Despite flatfishes being a well-known group that has been studied for centuries, many of its phylogenetic relationships are still debated. Discrepancies in the placement of Psettodidae as well as the relationships between many other clades may stem from inconsistencies between studies in taxon sample size, taxon representation, and which genetic markers are used. The issue is further complicated by apparent bursts of evolution early in the flatfish tree, potentially leading to incomplete lineage sorting (ILS) (Ribeiro et al. 2018), and extensive heterotachy in the form of both rate variation among lineages and base compositional heterogeneity, with many flatfish lineages having extremely long branches and extreme compositional heterogeneity (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, as has been done for the Pleuronectidae (Cooper & 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. Their analysis provided several novel topologies and recovered 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 (Figure 11). 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 & Ortí (2014) who sampled 23 genetic markers. Their 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) provides the only study that examines deep relationships within the Pleuronectiforms using a genome-wide dataset derived from high-throughput "next-generation" sequencing methods. Their study 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 & Ortí (2014), indicating that further application of next-generation methods to more specious datasets may allow flatfish systematists to attain more consistent results or identify sources of uncertainty.

The exon-capture method developed by Li et al. (2013) is a phylogenomic approach that has been used to examine interspecific 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. In this study we explore the relationships of the pleuronectoid flatfishes using a genomic dataset of 4,434 markers and compare our results to those from other comprehensive molecular datasets – e.g., Betancur-R & Ortí (2014), Harrington et al. (2016), Byrne et al. (2018), Vinnikov et al. (2018). Vinnikov et al. (2018) in particular hypothesized a phylogeny of the Pleuronectidae with near complete taxon sampling. By also densely sampling taxa from this family, we can further test the reliability of the markers used here for deciphering more recent branches. This study aims to contribute by improving our understanding of relationships within flatfishes by expanding both the number of taxa and loci sequenced through an exon-capture approach with a focus on family-level relationships, the family Pleuronectidae, and mapping out regions of persistent uncertainty within the phylogeny.

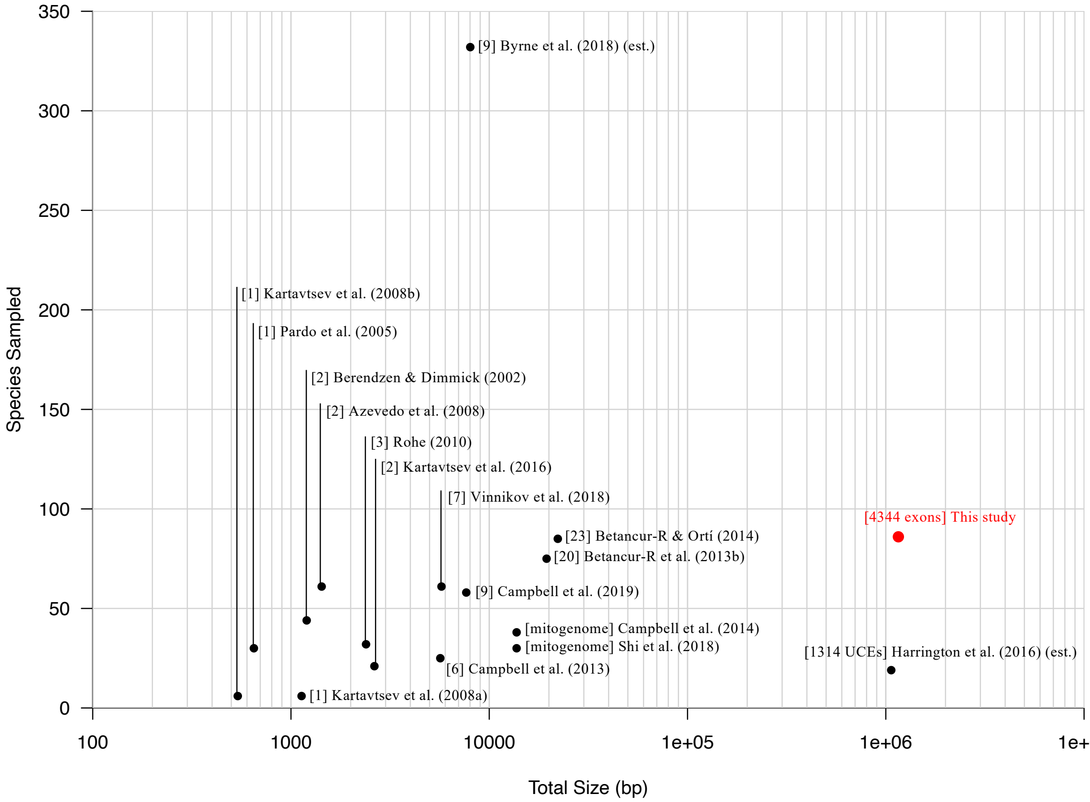


Figure 11: Sample sizes of taxa and genetic material from molecular phylogenetic datasets focusing on the flatfishes; "Species sampled" represents the number of flatfish (Pleuronectiformes) species included and "Total size" shows the combined length of all sequenced regions in base pairs (bp) along a logarithmic scale. The number of markers is shown in brackets. (est.) = "Total size" was estimated; exact value unknown.

Methods

Frozen fin and muscle tissues were sub-sampled from 96 specimens representing 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) and the remaining Pleuronectoidei being a well-supported monophyletic group (Norman 1934, Hensley and Ahlstrom, 1984, Chapleau 1993, Berendzen & Dimmick 2002, BHC model).

Specimens were sourced from fish collections (University of Washington Fish Collection, UWFC; Kansas University, KU; Commonwealth Scientific and Industrial Research Organisation, CSIRO; Louisiana State University, LSU) and from trawl surveys conducted by the National Oceanic and Atmospheric Administration (NOAA) Alaska Fisheries Science Center (AFSC) and the Northeast Fisheries Science Center (NEFSC). DNA was extracted using the DNeasy Blood & 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 but is sensitive enough to distinguish species level differences (Kuang et al. 2018, Song et al. 2017, Li 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, 2020). 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 an attempt 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) 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 Molecular 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 & Genotyping Center on two lanes of Illumina HiSeq 2500 System using paired-end 150 bp reads.

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 a single contig iteratively for each gene and sample using String Graph Assembler (SGA) (Bauer et al. 2011), then further assembled by the Assexon perl script merge.pl, utilizing alignment positions generated by Exonerate (Slater & Birney 2005). Potential paralogues were identified and removed by finding the best reciprocal hits between assembled contigs and a reference genome of *Oreochromis niloticus* with the perl script reblast.pl. A total of 4,378 of the 4,434 targeted markers were recovered in at least one sample. All samples were represented by more than 1,000 genes. Genes with high levels of missing data (i.e. no data for at least 70% of the total number of taxa) were removed. All 4,378 exons were aligned using MAFFT (Katoh et al 2002), and after removing 191 poorly aligned markers, a total of 4,187 exon markers were used for downstream phylogenetic analysis.

We reconstructed phylogenies using two methods. In the first method, which was a concatenation-based approach, we aligned genes for all taxa and loci were combined into one supermatrix. Then using this master gene data set, we estimated a maximum likelihood (ML) tree using RAxML (Stamatakis 2014) with 100 bootstrap (BS) iterations under the GTRCAT model (suited for fast calculation and getting better likelihood values if sample size is greater than 50, Stamatakis 2006). In the second method, we first inferred ML trees for each gene alignment in RAxML using the same settings. Then, a coalescent-based species-tree was inferred from all gene trees using ASTRAL-III (Zhang et al. 2018). Support was calculated as the Local Posterior Probability (PP) (Sayyari & Siavash 2016). Both concatenation and species-tree based methods were applied to our dataset consisting of all genes (with completeness level >70% of samples).

A second filtered dataset was obtained by screening for clocklike loci using the unfiltered concatenation-based tree as a reference. Filtering for only the most clocklike loci is a technique that aims to remove loci that have complex patterns of nucleotide evolution across taxa in the phylogeny, which may signify several factors that could cause gene-tree species-tree discordance (i.e. unintended presence of paralogs, 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) The clocklikeness test produces and compares likelihood values for a ML tree with and without a molecular clock constraint for each gene, using a ratio between the values as a proxy for molecular clocklikeness (MCL). We found the ratio of likelihood values calculated in PAUP (Swofford 2003) to correlate with gene length, making the test favor smaller genes with less information, so we followed Kuang et al.

(2018) and created a modified MCL by dividing the original ratio by the gene's length. Our complete dataset was screened to include only those genes with a modified MCL ratio less than 0.8 or a length greater than 500bp. This resulted in a filtered dataset consisting of 566 genes. We once again used a concatenation-based approach with RAxML and a species-tree approach with ASTRAL to construct phylogenies using this filtered dataset.

Results

The four different phylogenetic analyses resulted in trees with slightly different topologies and varying levels of support (Table 1). Based on the 94 nodes that were evaluated, the unfiltered-concatenation-based species tree had the highest overall support. The average support values decreased when using the clocklike-filtered dataset and the species-tree method. From the unfiltered dataset, low support values (BS<80; PP<0.80) were detected for nodes near the base of both trees. These nodes represent positioning of the families Achiridae, Poecilopsettidae, and Scophthalmidae that were not found in the other three trees. The analysis that achieved the highest resolution for these basal branches in the phylogeny was the concatenated approach on the clocklike-filtered data, in which BS=100 for nearly all above-family relationships. Instead, uncertainty in this tree was high mainly within the Pleuronectidae. The clocklike-species-tree exhibits the least overall support, and uncertainty is widely distributed throughout the phylogeny.

Table 1: Summary of gene datasets and methods of phylogenetic tree construction.

Dataset	**Avg. p-dist.**	**Method**	**Avg. support (94 nodes)**
Unfiltered 4,187 genes total size = 3,302,187 bp avg. size= 224.68 ± 213.34 bp	0.072 ± 0.026	Concatenation	BS = 97.4 (< 100 in 10 nodes)
		Species-Tree	PP = 0.967 (< 1 in 10 nodes)
Clocklike 566 genes total size = 215,286 bp avg. size = 380.36 ± 397.77 bp	0.065 ± 0.022	Concatenation	BS = 96.1 (< 100 in 15 nodes)
		Species-Tree	PP = 0.926 (< 1 in 29 nodes)

Family-level groups were all recovered as strongly monophyletic (BS=100; PP=1) with the exception of Pleuronectidae. Pleuronectidae was recovered as monophyletic in three of four phylogenies, but with low support in two (BS=65; PP=0.73). Pleuronectidae monophyly was not supported in the species-tree analysis of the clocklike dataset, where the pleuronectid genus *Atheresthes* is sister to a clade containing both the Paralichthyidae (*sensu* Campbell et al. 2019) and the remaining Pleuronectidae genera, but with very low support (BS=62).

The overall family-level topology of our dataset remained fairly stable between methods of tree inference and sets of genes. Citharidae invariably resolved as sister to all other Pleuronectoidei, and all topologies contained a strongly supported monophyletic group consisting of five families: Rhombosoleidae, Cyclopsettidae, Bothidae, Paralichthyidae, and Pleuronectidae. Additionally, a second group consisted of Samaridae, Soleidae, and Cynoglossidae, with less strongly supported inclusion of Poecilopsettidae and Achiridae (Figures 12 & 13).

The families Poecilopsettidae, Scophthalmidae, and Achiridae were each found in strongly supported positions within three phylogenies and were poorly supported in the remaining tree. Poecilopsettidae was sister to the Soleidae-Cynoglossidae clade (BS=100; PP=0.91) in three phylogenies, but its position was unresolved in the unfiltered-species-tree (PP=0.46). In three analyses, Scophthalmidaewas sister to the Rhombosoleidae-Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae clade (inclusive of Poecilopsettidae in the unfiltered-species-tree) (BS=100; PP=1, 0.86), but its position was unresolved in the unfiltered-concatenation-based phylogeny (BS=26). In three phylogenies, Achiridae was sister to the Samaridae-Soleidae-Cynoglossidae (inclusive of Poecilopsettidae in two phylogenies) (BS=100;

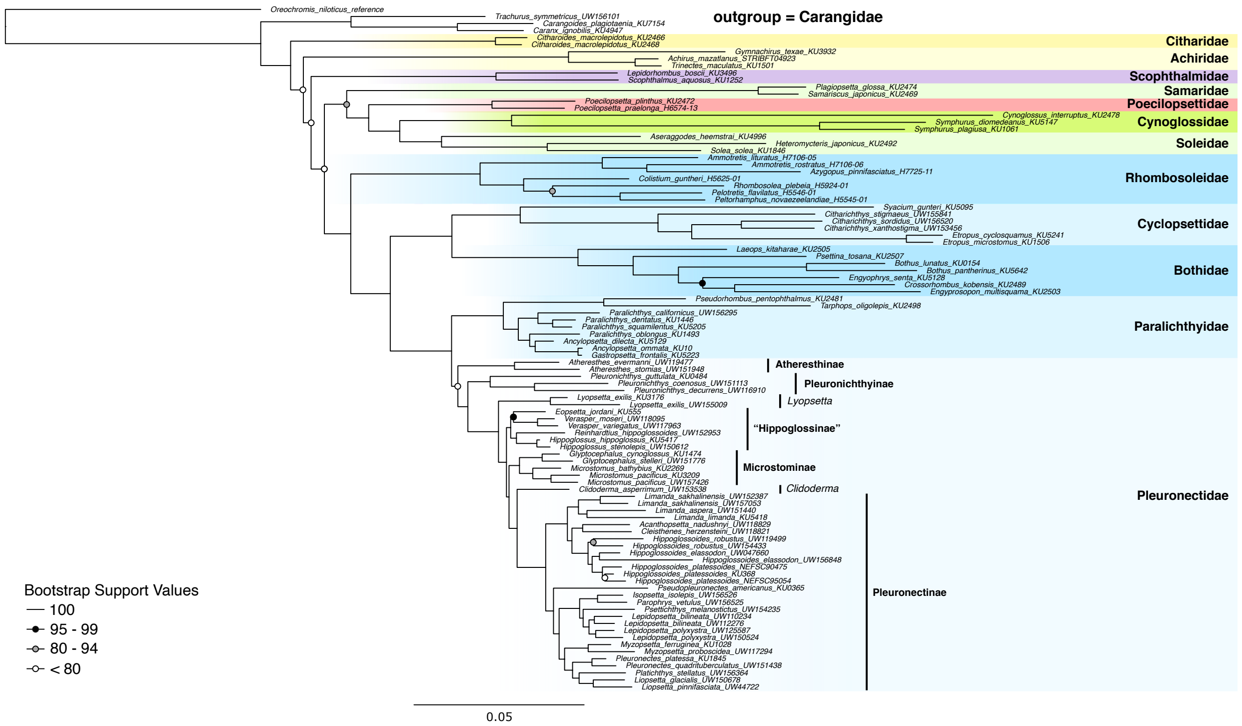
PP=1, 0.99), but in the unfiltered-concatenation-based tree the family was poorly supported as sister to all other Pleuronectoidei except Citharidae (BS=63).

Of the five Pleuronectidae subfamilies (Atheresthinae, Pleuronichthyinae, Hippoglossinae, Microstominae, and Pleuronectinae), our data strongly support the monophyly of all except the Hippoglossinae, for which we recovered three groups: one each for the monotypic genera *Lyopsetta* and *Clidoderma*, and one containing the genera *Eopsetta*, *Verasper*, *Reinhardtius*, and *Hippoglossus*. All six genera were only found to be united in our clocklike-concatenation tree (BS=92). The analyses found spurious relationships between the four genera of the third group, as there were three unique topologies among the four trees and all trees were poorly supported. Additionally, we were unable to resolve the relationships between the three groups, Microstominae, and Pleuronectinae.

Additional intergeneric uncertainty was found in the Rhombosoleidae and Bothidae. Within Rhombosoleidae, we were unable to determine if *Rhombosolea* or *Colistium* was more closely related to *Pelotretis* and *Peltorhamphus*. In Bothidae, the relationships among species varied substantially between analyses. The most well-supported topology was that of the unfiltered-concatenation-based species tree which shares its topology the other unfiltered tree.

Four genera were recovered as non-monophyletic: *Ammotretis* (Rhombosoleidae), *Ancylopsetta* (Paralichthyidae), *Citharichthys* (Cyclopsettidae), and *Paralichthys* (Paralichthyidae). *Ammotretis* was paraphyletic containing *Azygopus*. *Ancylopsetta* also comprised representatives of *Gastropsetta*. Similarly, *Citharichthys* was resolved along other species of the genus *Etropus*. The genus *Citharichthys* was only resolved as monophyletic in the clocklike-concatenation-based species tree, but with low support (BS=78). A non-monophyletic topology was found for *Citharichthys* in all other trees and in all trees for the other three genera.

(a) unfiltered-concatenation-based



(b) unfiltered-species-tree

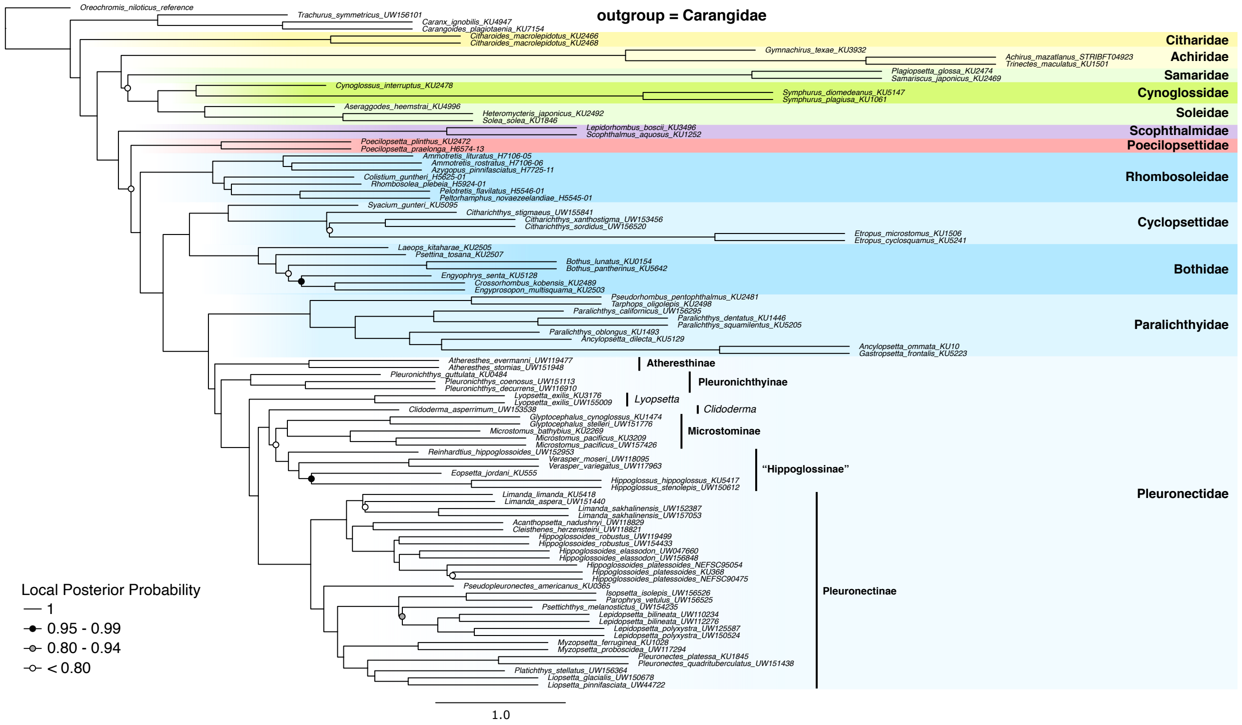
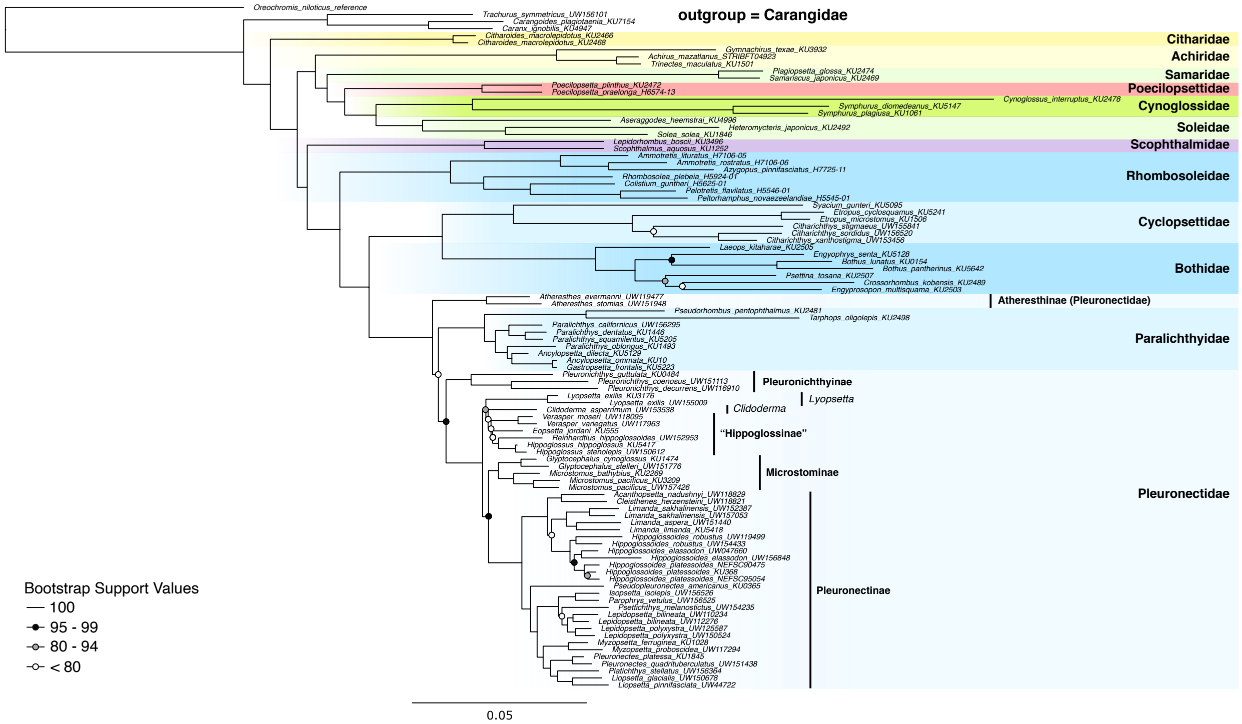


Figure 12: Maximum likelihood phylogenies generated from a total evidence dataset of 4,106 genes using (a) the concatenation-based method and (b) the species-tree method. "Hippoglossinae" = Hippoglossinae (*sensu* Vinnikov 2018) excluding *Clidoderma* and *Lyopsetta*. Exact support value can be found in Figure S1.

(a) clocklike-concatenation-based



(b) clocklike-species-tree

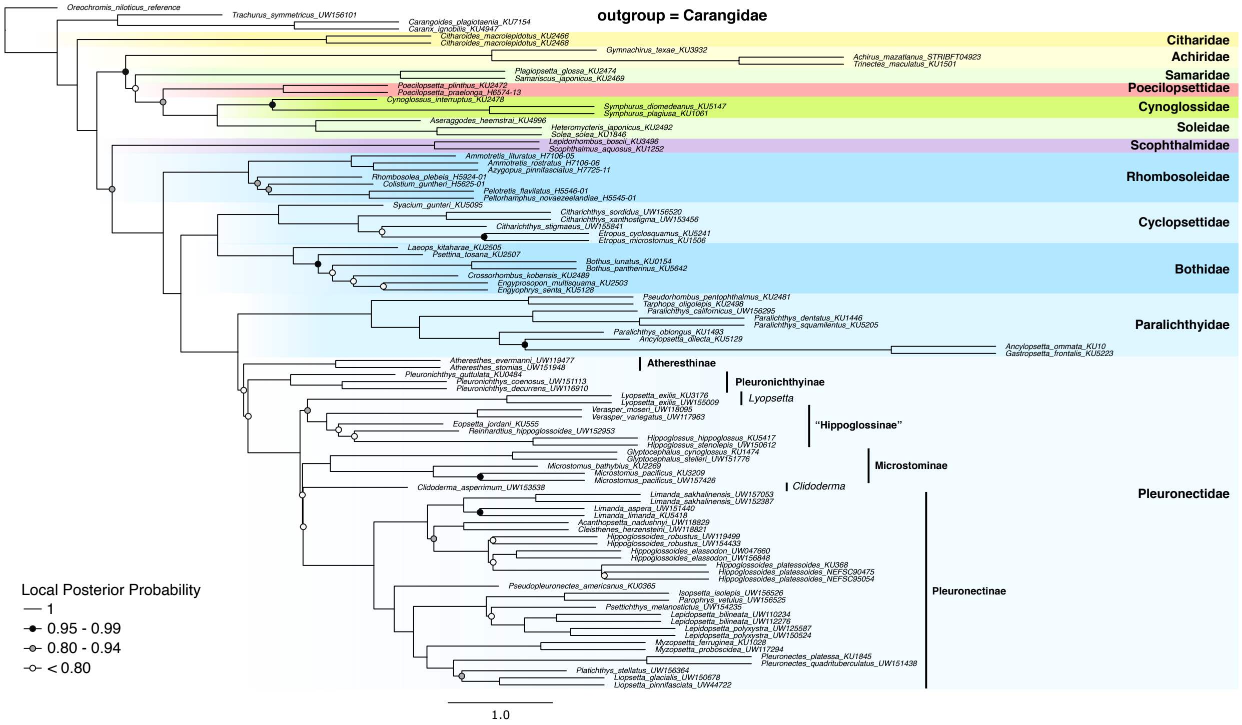


Figure 13: Maximum likelihood phylogenies generated from a filtered dataset of 566 genes screened for clocklikeness using (a) the concatenation-based method and (b) the species tree-method. "Hippoglossinae" = Hippoglossinae (*sensu* Vinnikov 2018) excluding *Clidoderma* and *Lyopsetta*. Exact support value can be found in Figure S1.

Discussion

By using the exon-capture method we aimed to expand upon the previous and only genome-wide dataset focusing on flatfishes (Harrington et al. 2016) to infer evolutionary relationships with greater genomic and taxonomic coverage. Disagreement between the many phylogenetic hypotheses that have been proposed can be attributed to studies using datasets with a number of genetic markers and taxa. In their analysis of >1200 UCEs, Harrington et al. (2016) were able to replicate the topology between 13 of 15 families in Betancur-R & Ortí (2014), the largest Sanger dataset (using 23 genes). Both UCEs and exons have been used to infer phylogenies in other organisms (Wood et al. 2018, Bossert et al. 2019), but are different in nature, i.e. UCEs are under highly selective pressure and have more complex patterns of variability, whereas most of the variation in exons is restricted to the third codon position (McCormack et al. 2012, Bragg et al. 2016). Our exon-based results show that while some relationships within the Pleuronectiformes are remarkably consistent across analyses, even with extensive sampling from across the genome, sequence data still fail to provide cohesive evidence for a single well-supported phylogenetic hypothesis. Different topologies and levels of uncertainty were achieved by using different tree construction methods and applying them to different sets of genetic data.

Gene filtering

Independent of tree-reconstruction method, the full dataset of 4,157 markers resulted in more highly supported relationships than with the clocklike-filtered dataset (Table 1). Filtering for clocklikeness was done 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). Our clocklike dataset produced more poorly supported topologies near the tips of the phylogeny, possibly due to a smaller amount of data overall, but the concatenated analysis recovered strongly supported relationships near the base of the flatfish tree. Between the concatenated and species-tree methods, the clocklike trees were more dissimilar to one another other than to those from the unfiltered dataset, indicating the clocklike dataset was also more susceptible to bias associated with the tree-reconstruction method, possibly from applying an excessively narrow filter. The 566 clocklike genes represent 7% of the genetic material from the unfiltered dataset. Poor support in more recent branches may also indicate that fast-evolving genes that could have informed relationships towards the tips of the tree were determined to be non-clocklike and removed. Kuang et al. (2018) validated the usefulness of the MCL by showing high levels of similarity between the clocklike gene-trees and the reference tree; however, in our case the reference tree (Figure 12) contained several poorly supported nodes, which may have biased our filtering to select for loci that supported potentially incorrect topologies. While filtering of genome-scale datasets is highly encouraged (Phillips et al. 2004), it may be useful to explore other screening techniques based on criterion such as substitution rate (Nosenko et al. 2013), nucleotide composition (Phillips & Penny 2003), and phylogenetic informativeness (Townsend 2007, Lopez-Giraldez et al. 2013).

Concatenation and species-tree methods

With both filtered and unfiltered datasets, the concatenation approach resulted in more well-supported relationships, more notably in the clocklike-filtered dataset. Concatenation and species-tree methods are both commonly used in phylogenetic inference (Betancur-R & Ortí 2014, Harrington et al. 2016, Kuang et al. 2018, Li et al 2018) but differences in how each treats the data will affect resulting trees differently. In the concatenation approach, the result will be more heavily influenced by longer, faster evolving genes; however, due to the computational complexities of applying different substitution models, our concatenated alignment was predicted under a single model, which fails to account for variable rates of evolution among genes, and potentially creates a bias toward genes that are best predicted by the chosen model (Xi et al. 2015). The species-tree method is expected to reduce these gene-specific biases, especially from ILS (Liu et al 2009, Tonini et al. 2015), but because our dataset is comprised of mostly small genes (<300 bp), the resulting species-trees have higher rates of uncertainty. This is because the multispecies coalescent model used in ASTRAL assumes that the gene-trees informing the species tree are known without error and small genes provide little information to derive each gene-tree, especially those that are evolving slowly (Xi et al. 2015). This effect is compounded by a reduced number of genes in the clocklike-species-tree, even though the filtered dataset contained larger genes on average (Table 1). In summary, the species-tree relationships contain less error due to gene-specific bias but at the expense of uncertainty from small genes.

Family-level relationships of the Pleuronectiformes

Overall, our results show a pattern of increasing resolution with more data, but support for relationships varies somewhat depending on the analytical approach, and the size and characteristics of the dataset used, especially with regards to resolving relationships at differing evolutionary timescales. Most nodes at the family level were well supported in at least one of our trees, and usually more than one. Within the families Pleuronectidae and Bothidae, relationships were best supported by the unfiltered dataset (Figure 12). Still, the positions of several families and subfamilies remain elusive and are discussed in the context of previous studies below.

Four flatfish families were not examined in this study: Psettodidae, Paralichthodidae, Oncopteridae, and Achiropsettidae. Psettodidae is the only flatfish group excluded from the Pleuronectoidei (Berendzen & Dimmick 2002, Betancur-R et al. 2013b, Betancur-R & Ortí 2014, Harrington et al. 2016) and its placement within the broader Carangaria still remains uncertain (Li et al 2011, Near et al. 2013, Betancur-R et al. 2013a, Campbell et al. 2013, Shi et al. 2018). Campbell et al. (2019) found that Paralichthodidae, Oncopteridae, and Achiropsettidae are more closely related to Rhombosoleidae than to any other families. Currently, there is no strong counterevidence against a monophyletic Paralichthodidae-Oncopteridae-Achiropsettidae-Rhombosoleidae clade.

The evolutionary relationships of the BHC model were mostly replicated in this study. The BHC model only differs from our results in that Rhombosoleidae is positioned more proximal to the Achiridae than to any other family included in this study. This may be caused by our use of a larger molecular dataset compared to Sanger datasets. It is challenging to compare our position of Rhombosoleidae with the genome-scale phylogeny from Harrington et al. (2016) since Rhombosoleidae was excluded from their study. Instead, they included its sister family Achiropsettidae (Betancur-R et al. 2013b, Betancur-R & Ortí 2014, Byrne et al. 2018, Shi et al. 2018, Campbell et al. 2019), which was resolved as sister to Achiridae, suggesting a similar position for Rhombosoleidae that is still incongruent with our results. It is plausible that placement of the Rhombosoleidae is poorly resolved from sequence data and that the topology in this study is an artifact of using exons rather than UCEs and the specific set of markers included.

By cross referencing our results with the BHC model and previous analyses, we can identify parts of the flatfish phylogeny that are consistently strongly supported and areas where there is persistent uncertainty. While the monophyletic status of the Pleuronectiformes has mixed support, its division into the two suborders Psettoidei (*sensu* Regan 1910) and Pleuronectoidei (*sensu* Chapleau 1993) is widely accepted. Within the Pleuronectoidei, the family Citharidae typically resolves as sister to all other groups (BHC model). Campbell et al. (2019) refers to this lineage as the superfamily Citharoidea. Our analyses support this position, but strong support for a monophyly between its six species has been and remains elusive (Regan 1910, Amaoka 1972, Hensley & Ahlstrom 1984, Aboussouan 1988, Chapleau 1993, Cooper & Chapleau 1998a, Byrne et al. 2018, Shi et al. 2018) and the Achiridae are occasionally placed within this early branch of the tree (Byrne et al. 2018, Shi et al. 2018, Azevedo et al. 2008) with mixed support. This position has only been recovered in studies that heavily sample from the mitochondrial genome, and in those studies, the Citharidae does not resolve as monophyletic. It may also be an artifact of long branch attraction as both the Achiridae and Citharidae diverged near the base of the Pleironectoidei, which may have allowed for the accumulation of homoplasies. The sister branch to the Citharidae leads to a highly unresolved region of the phylogeny relating the remaining families. This region represents different topologies from different studies; some are well supported, but in most cases they are more poorly supported and characterized by short branches, likely caused by rapid radiation early on in flatfish evolution. Emerging from this polytomy, there are five lineages that are fairly well supported: (1) the Achiridae clade, (2) the Samaridae-Poecilopsttidae-Cynoglossodae-Soleidae clade, (3) the Paralichthodidae-Oncopteridae-Achiropsettidae-Rhombosoleidae clade, (4) the Scophthalmidae clade, and (5) the Cyclopsettidae-Bothidae-Paralichthyidae-Pleuronectidae clade.

In addition to being sister to the Citharidae, the Achiridae has historically been recovered 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.

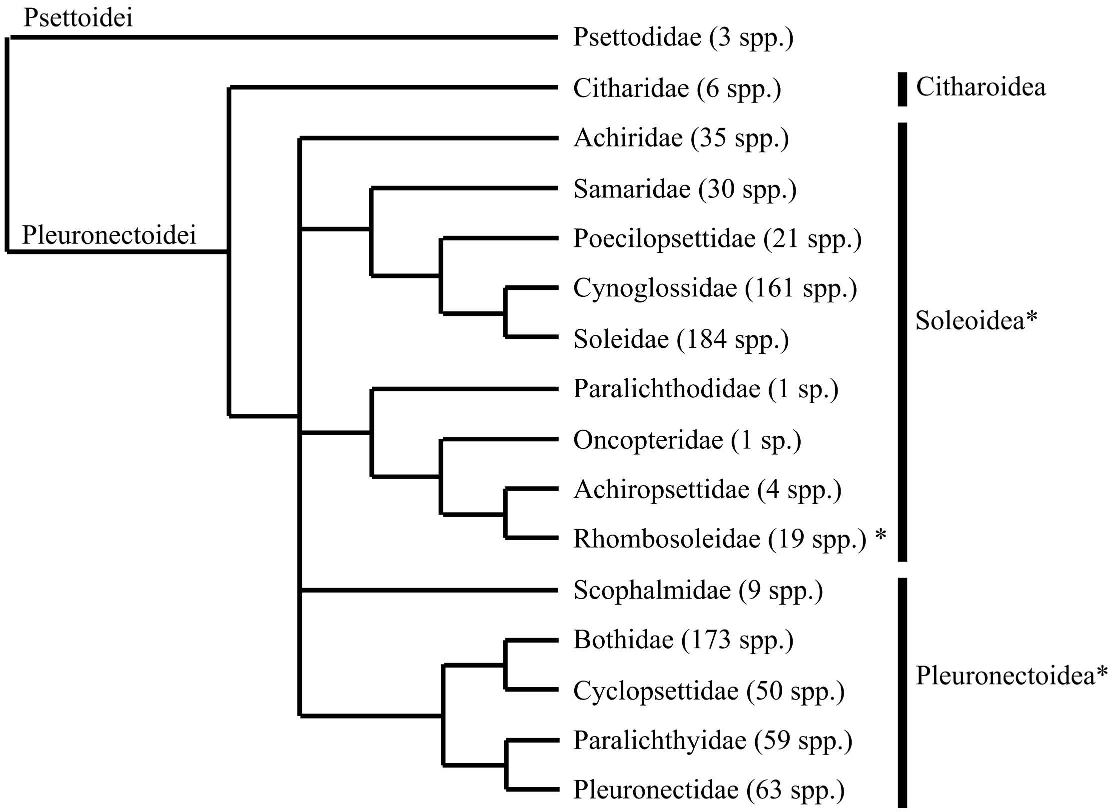


Figure 14: Conceptual phylogeny of the Pleuronectiformes families based on consensus between the BHC model and this study. (*) indicates likely non-monophyletic; Rhombosoleidae may not be monophyletic if *Psammodiscus* is included; Soleoidea and Pleuronectoidea are poorly supported taxonomic groups.

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 contentions. Poecilopsettidae has appeared in several other places in the broader Pleuronectiformes tree (Berendzen & 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 *P. 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 lineage recovered in previously mentioned studies (BHC model) and includes the genus *Nematops* (Campbell et al. 2019), however, the validity of these two lineages is dubious. Shi et al. (2018) recovered *P. natalensis*, part of the group two lineage, in a clade with the Cynoglossidae, Soleidae, and Samaridae, the same complex formed by the group one position. 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 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.

Phylogenetic inference of the Rhombosoleidae and its phylogenetically proximal lineages (Achiropsettidae, Oncopteridae, Paralichthodidae) is 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*, *Taratretis* This study is the only molecular phylogeny to our knowledge that contains *Azygopus*. Our analysis recovered 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 these five families as the superfamily Soleoidea. Our analysis does not support a monophyletic Soleoidea.

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 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 (Figures 12 & 13), casting doubt on a monophyletic Pleuronectoidea. The group containing all six of these families superficially resembles the "bothoid" group (*sensu* Hensley & Ahlstrom 1984) defined as the Bothidae, Paralichthyidae (excluding *Tephrinectes* and *Thysanopsetta*, but including the Cyclopsettidae), Pleuronectidae (*sensu* Norman 1934, which includes Rhombosoleidae, Samaridae, and Poecilopsettidae), and *Brachypleura* because they all share a unique caudal skeleton. This group has largely been shown to not be monophyletic, but the contemporary model of the flatfish phylogeny would suggest that the "bothoid" caudal skeleton had appeared early on in the flatfish tree and was modified in several lineages such as in several Citharidae, Achiridae, and the Soleidae-Cynoglossidae clade. In our phylogenies, the Scophthalmidae lineage also appears as more distantly related to the other four Pleuronectoidea families than the Poecilopsettidae (Figure 2), and Soleidae, Cynoglossidae, and Samaridae (Figure 2a) depending on the tree construction method, and datasets of primarily mitochondrial genes have produced topologies with Scophthalmidae being the sister group to the Rhombosoleidae (Byrne et al. 2018, Shi et al. 2018).

Species relationships of the Pleuronectidae

Pleuronectidae is the most densely sampled family in our analysis (38 of 61 species in 23 of 24 genera). The results from our study are consistent with the overall topology found by

Vinnikov et al. (2018) with a few exceptions. We show that *Lyopsetta* and *Clidoderma* should not be included in the Hippoglossinae since this region of the tree is poorly supported and fails to resolve in one singular topology (Figures 12 & 13). Vinnikov (2018) justified a monophyletic Hippoglossinae by finding the group united in all gene trees, however, this lineage has low posterior support in their concatenation-based tree. Furthermore, our analysis indicates that getting further resolution on the relationships between *Lyopsetta, Clidoderma,* the remaining Hippoglossinae, Microstominae, and Pleuronectidae is dubious. Collective inconsistencies and poor node support from several studies including this one suggests there may be additional persistent uncertainty among the remaining four Hippoglossinae genera (Vinnikov et al. 2018), in the Pleuronectinae between *Hippoglossoides*, *Limanda*, and the clade containing *Cleisthenes*, *Acanthopsetta*, and *Dexistes* (Roje 2010, Vinnikov et al. 2018), and between *Psettichthys*, *Lepidopsetta*, and the clade containing *Isopsetta* and *Parophrys* (Vinnikov et al. 2018). There is one pleuronectid genus, *Myzopsetta*, that may not be monophyletic. *Myzopsetta* was merged into the genus *Limanda* (Evseenko 2003) but was resurrected by Busby et al. (2017) on the basis of larval characteristics. Molecular phylogenies have supported re-separating these genera (Roje 2010, Kartavtsev et al. 2016, Ji et al. 2016, Vinnikov et al. 2018), but Vinnikov et al. (2018) found two lineages within this group, one more closely related to *Pleuronectes*, *Platichthys*, and *Liopsetta*. Further investigation is required to determine if *Myzopsetta* should be split into two genera. Suzuki et al (2001) had questioned the monophyly of Pleuronectidae on the basis of the placement of *Atheresthes*. Our data show that the origin of the *Atheresthes* lineage is rooted near the most recent common ancestor (MRCA) between Pleuronectidae and Paralichthyidae and that its position is sensitive to phylogenetic methodology, but most studies report inclusion of *Atheresthes* in Pleuronectidae (Betancur-R & Ortí 2014, Vinnikov et al. 2018, Byrne et al. 2018).

The unstable position of the *Atheresthes* branch could be further explained by limited genetic data in Suzuki et al. (2001) and very long branch lengths in this study.

Remaining knowledge-gaps in the flatfish phylogeny

There have been numerous order-level molecular phylogenies that have attempted to resolve relationships between flatfish species (BHC model, Berendzen & Dimmick 2002, Azevedo et al. 2008, Byrne et al. 2018, Shi et al. 2018, this study), all are subject to problems of incomplete taxon sampling and discordant genetic information. While the general structure between families is consistently replicated, the sister clade to the Citharidae continuously fails to resolve any further (polytomy in Figure 14), even with the genomic-wide dataset used in this study. The molecular 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 2.

Table 2: 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. 2020, van der Laan & Fricke 2020). Questionably monophyletic genera have been recovered 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	173	No; most extensive analysis: Fukui (1997) & 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 & Ahlstrom (1984) & Khidir et al. (2005)	*Citharichthys* (Betancur 2014, Byrne et al. 2018, this study), *Etropus* (Byrne et al. 2018); [2/4]	none
Cynoglossidae	3	161	No, need a focus on monophyly of *Cynoglossus*; most extensive analysis: Cooper & 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)	*Myzopsetta* (Vinnikov et al. 2018); [1/16]	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	19	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	184	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*

Conclusion

Our analysis of flatfish systematics using an exon-capture dataset with relatively dense taxon and genetic sampling showed remarkable consistencies with the BHC model and the Pleuronectidae species relationships from Vinnikov et al. (2018), even when different sets of genes and tree-reconstruction methods were applied. Still, even with a large molecular dataset, several relationships at the family and intrafamilial-level fail to resolve, likely representing periods of rapid evolution and extensive heterotachy among flatfish lineages. By comparing our results with other flatfish phylogenies we were able to identify several regions where phylogenetic uncertainty is likely to remain and outline groups that should be targeted for further study.

Chapter 2: **Species delimitation in four putative species pairs of North Pacific flatfishes**

Contributions:

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Abstract

Flatfishes of the family Pleuronectidae are widely abundant in the North Pacific and contribute heavily to commercial fisheries. Management strategies are more likely to succeed if species definitions reflect lineages that are naturally separated by reproductive boundaries, however, most managed flatfish species that have been known for a long time were only described using morphological characters and there is insubstantial genetic evidence to justify their existence. We examined four species pairs known from Alaska, one each from the genera *Atheresthes, Hippoglossoides, Lepidopsetta,* and *Limanda*, and performed several species delimitation analyses (phylogenetic species, multispecies coalescence, genetic distance, and admixture coefficients) using exon-capture data to provide a genetic framework to assess their utility in the context of management. For each species pair, there were variable levels of support across methods. Overall, *Lepidopsetta* and *Limanda* species are well-supported evolutionary lineages with reliable diagnostic morphologies, and thus are good management species. *Hippoglossoides* species were also supported as distinct lineages, but there was evidence of high rates of genetic exchange across lineages, possibly caused by recent or incomplete speciation. *Atheresthes* species failed to resolve as distinct lineages in all analyses, but admixture analysis found potential for the existence of two populations that were poorly correlated with species identifications.

Introduction

Flatfishes (Pleuronectiformes) are a large constituent of the marine fish community in the North Pacific. There is extensive documentation on North Pacific flatfishes, in part due to their large contribution to local fisheries. In Alaska, there are 29 species of flatfishes in the family Pleuronectidae that span a range of trophic niches (Mecklenburg et al. 2002). These species are regularly surveyed and their harvest is regulated by the National Oceanic and Atmospheric Administration (NOAA).

Defining species is a critical foundation for any stock assessments, predictive models, and implementing management strategies. In order for management efforts to be effective, it is vital that a species reflects a group of individuals that are commonly interbreeding in nature and capable of reproducing with one another to replenish and maintain the group. Measuring patterns of interbreeding is particularly challenging in broadcast-spawning species such as flatfishes, so one way to identify such groups indirectly is by finding genetically similar clusters of individuals representing evolutionarily distinct lineages (i.e. species). Once these genetic species are established, effective management also relies on the ability to quickly diagnose species in the field during surveys, so genetically identified species should then be described using morphological characters that reliably differentiate them from other species. In practice, correlation between the defining morphological and genetic characters of a species is often poor or unknown.

Nearly all Alaskan flatfishes were described prior to genetic sequencing techniques, and were therefore based solely on morphological characteristics. This can be problematic for fisheries management if such species do not represent evolutionarily distinct lineages. If putative species are comprised of more than one lineage, then our estimates of the total population abundance of each species would be overestimated. There can also be cases of excessive taxonomic splitting, where morphological differences are phenotypic, geographic, and/or ontogenetic variation rather than diagnostic for evolutionarily distinct species. Furthermore, identifications from these poorly defined species become preserved in historical records, limiting their scientific utility.

Of the 29 pleuronectid flatfishes in Alaska, there are four species pairs, one each from the genera *Atheresthes, Hippoglossoides, Lepidopsetta,* and *Limanda,* that are of particular interest to explore their validity as distinct species from a genetic basis. These are the only flatfish genera with more than one species occurring in Alaska. All eight species were described based on morphology and genetic studies provide insufficient sampling to infer species definitions (Vinnikov et al. 2018). Each pair contains species that are morphologically and ecologically similar to one another, are likely candidates for being sister species, and have different but largely overlapping geographical ranges. The range overlap in all four cases occurs in the Bering Sea, which may be an indication that these species are being affected by similar evolutionary processes, possibly associated with fluctuating sea-ice dynamics and periodic flooding of the continental shelf.

The two species of *Atheresthes* have relatively large mouths with long teeth, are often caught swimming hundreds of meters above the sea floor, and employ a self-destructive defensive strategy, secreting a muscle dissolving enzyme when under stress that historically rendered them inedible after being caught (Reppond et al. 1993). *Atheresthes evermanni* (Kamchatka flounder) is distinguished from *A. stomias* (arrow-tooth flounder) by the number of gill rakers on the 1st gill arch (< 14 in *A. evermanni* and > 14 in *A. stomias*), 2nd upper gill arch (1 in *A. evermanni* and 2 or 3 in *A. stomias*), and position of the dorsal eye (which extends over the dorsal margin in *A. stomias*, making it visible from the blind-side) (Mecklenburg et al. 2002). Although both species are piscivorous as juveniles and adults (Yang & Livingston 1986), the juveniles of *A. stomias* consume a disproportionately higher number of euphausiids. *Atheresthes stomias* has a large range extending from the Bering Sea southward to California whereas *A. evermanni* has a range extending westward from the eastern Aleutian Islands into the Sea of Okhotsk and northward into the Chukchi Sea with the Bering Sea as an area of sympatry. Although the sampled range of early life history is limited, the larval distribution of *A. stomias* in Alaska is largely centered in the Gulf of Alaska, while *A. evermanni* larvae are mainly found in the southern Bering Sea. The early life stages are discernable as *A. evermanni* develops pigmentation more quickly, but between 12-21mm postflexion larvae of both species exhibit have similar pigmentation (De Forest et al. 2014). Attempts to identify diagnostic characters at this stage have not been successful (De Forest et al. 2014).

The *Hippoglossoides* species found in Alaska are *H. elassodon* (flathead sole) and *H. robustus* (Bering flounder). The genus also contains two other species, *H. dubias* found along the Asian coast of the North Pacific, and *H. platessoides* in the North Atlantic. The two Alaskan species are differentiated mainly using gill raker counts on the lower limb of the 1st gill arch (usually > 14 in *H. elassodon* and < 13 in *H. robustus*) (Mecklenburg et al. 2002). Additionally, *H. elassodon* has stronger lateral compression of the body and has a more pronounced interorbital ridge with fewer scales, and in life, *H. elassodon* has a blotchier pattern than *H. robustus*, especially on the fins. As with *A. stomias*, *H. elassodon* has a large range extending from the Bering Sea and Aleutian Islands to California where as *H. robustus* is a more northern species, ranging from the Bering Sea to the Beaufort Sea. *Hippoglossoides robustus* is mainly known from areas with extensive continental shelf (<400m) while *H. elassodon*, has been caught along the continental slope up to 1050m deep. Larval *Hippoglossoides* are known from the Gulf of Alaska, throughout the Bering Sea, and into the Chukchi Sea, but early life stages are poorly described for *H. robustus*. Genetic-based studies have failed to resolve these species as reciprocally monophyletic lineages (Kartavtsev et al. 2007, 2008a, Vinnikov et al. 2018).

Lepidopsetta is a common genus of flatfish along the US West coast. What used to be considered a single species, *L. bilineata,* was reclassified as two, *L. bilineata* (southern rock sole) and *L. polyxystra* (northern rock sole) (Orr & Matarese 2000). The two species can be distinguished from one another based on 1st arch gill raker counts (usually < 10 in *L. bilineata* and > 10 in *L. polyxystra*), supraorbital pores (usually < 4 in *L. bilineata* and > 4 in *L. polyxystra*), the interorbital space (narrow in *L. bilineata* and wide in *L. polyxystra*), and blind side coloration in life (uniformly creamy white in *L. polyxystra* and white with glossy highlights and muscle bands in *L. bilineata*) (Orr & Matarese 2000; Mecklenburg et al. 2002). *Lepidopsetta bilineata* can be found as far south as California. The more northern *L. polyxystra* ranges into the Bering Sea and across the Aleutian islands, into the Sea of Okhotsk. A third species *L. mochigarei* is known from the western North Pacific. Both North American species feed mainly on benthic invertebrates but the diet of *L. bilineata* is mostly *Ophiura* brittlestars, while that of *L. bilineata* primarily contains various worms (Orr & Matarese 2000). These species have the strongest separation in adult distribution, however, the ranges of the early life stages overlap, particularly in the Gulf of Alaska.

Limanda aspera (yellowfin sole) and *L. sakhalinensis* (Sakhalin sole) are the only *Limanda* species in the North Pacific. A third species *L. limanda* is known from the North Atlantic. *Myzopsetta proboscidea* (Longhead dab) is another North Pacific species that was historically recognized within *Limanda* but was recently reclassified due to poor morphological and phylogenetic association with the other *Limanda* species (Busby et al. 2017, Vinnikov et al. 2018). The most notable distinguishing character for the two Alaskan *Limanda* species is the number of spinuals on eyed-side scales. In *L. sakhalinensis* nearly all scales are ctenoid and have 3-10 spinuals, while *L. aspera* scales are cycloid or ctenoid with 2 (rarely 3) spinuals. *Limanda sakhalinensis* can also be more elongate than *L. aspera* in both adults and larvae, and in life *Limanda aspera* has distinctive yellow median fins with a black line at the base of the dorsal and anal fins and a white blind-side, while *L. sakhalinensis* has brown fins and an off-white blind-side (Mecklenburg et al. 2002). The ranges of the two species are fairly similar, mostly inhabiting the Bering Sea, with *L. sakhalinensis* being less abundant and more associated with the western North Pacific to the Sea of Okhotsk. Both *Lepidopsetta* and *Limanda* live in shallower water (<700m), with *Lepidopsetta* found more frequently at depth (often caught down to 200m, while *Limanda* are rarely seen below 100m).

The power to delimit species using genetic data is dependent on both the type and amount of data used, coupled with the extent of genetic differentiation (i.e. time since divergence) between the lineages in question. It is unlikely that the variability of any one character will reflect patterns of evolution at the species scale if species are very recently diverged. One of the most widely applied methods of species identification using genetics is DNA barcoding of the mitochondrial cytochrome c oxidase-I (COI) gene using the Sanger method (Sanger et al. 1977). The function and location of COI is largely conserved in a wide variety of organisms, yet is variable enough to differentiate most species, with sequence similarity typically less than 98% between fish species (Ivanova et al. 2007, Ward 2009, Coker et al. 2018). In some taxa, however, COI alone is insufficient to delimit distinct evolutionary lineages (Liu et al. 2017). This is likely to be the case in closely related species that diverged recently, such as the flatfishes in this study.

Sampling a range of loci throughout the genome can reduce the effects from evolutionary patterns specific to COI (Liu et al. 2017). Many studies have incorporated a variety of genes in their Sanger datasets (Betancur-R & Ortí 2014, Byrne et al. 2018, Campbell et al. 2019) but are limited by the multiplicative labor cost for each additional gene. Next-generation sequencing techniques such as restriction site-associated DNA sequencing (RADseq) and target-capture are capable of sequencing thousands of loci with a relatively small labor cost. RADseq datasets are comprised of single nucleotide polymorphisms (SNPs) from DNA fragments determined by cleaving the genome at all instances of a restriction-site sequence (Baird et al. 2008). The advantage of this method is that we are not introducing bias from specific types of genomic DNA other than those associated with the restriction sites themselves. Examining SNP data can reduce computational workload by only using variable sites, but in turn information about the frequency of these sites is lost. Furthermore, in order to have comparable RADseq data across samples, the samples must be closely related enough that they have similar sets of restriction-sites, so RADseq is limited to shallower evolutionary timescales.

Alternatively, using a target-capture method (also known as hybrid enrichment, target enrichment or gene capture), specific loci are targeted using capture probes designed from annotated genomes of model organisms, to sequence their orthologs in samples of non-model organisms. Markers and protocols can be refined to work with a specific range of taxa (Li et al. 2013; Jiang et al. 2019), thus, this method can be used to infer both shallow and deep relationships. Many target-capture studies choose to target exons because they are among the most well-documented parts of genomes, and are conserved across a large number of organisms but also contain enough information to distinguish species-level differences (Kuang et al. 2018, Song et al. 2017, Li et al. 2018). With exon-capture data there is also the potential to compare and combine data across studies as there may be orthologous loci sampled independently in different datasets, whereas in RADseq, each dataset is more specific to the targeted taxa. While RADseq and exon-capture are both effective at capturing genome-wide variation, they are limited by short fragments that make each locus less informative than those used in Sanger sequencing. This study will use exon-capture data due to its reliability at both shallow and deep relationships, and because of the availability of existing markers already refined for ray-finned fishes (Jiang et al. 2019), thus ensuring compatibility with other exon-capture datasets.

There are many criteria we can use to infer distinct lineages based on genome-wide data from a group of sampled individuals. It is important that we consider multiple lines of evidence, as different methods will have different levels of sensitivity and make different assumptions about the data (Carstens et al. 2013).

One way to summarize the relationships between samples is with a phylogenetic analysis. If a species represents a distinct evolutionary lineage where enough time has passed following the last speciation event, then all individuals of that species should be recovered within a single united clade that excludes all other lineages (a monophyletic group), since all individuals descending from the common ancestor following the speciation event would possess genetic synapomorphies. Similarly, if one of our species pairs represents two distinct evolutionary lineages, then they should be recovered as reciprocally monophyletic groups – i.e. both species in the pair are monophyletic; however, non-monophyly is not necessarily evidence against a valid species and is more likely to occur if species diverged recently (i.e. not enough time for synapomorphies to evolve) or if the sampled genetic data do not reflect the evolutionary history of the species (e.g. due to incomplete lineage sorting, or hybridization/introgression). Furthermore, if there is extensive missing data for a given sample, it may appear to be more distantly related due to being more genetically dissimilar. The power of a phylogenetic analysis is highly dependent on the number of synapomorphies captured in your genetic sampling, relative to other mutations that are not phylogenetically informative. With exon-capture data, we can infer phylogenetic relationships based on all loci collectively or on a gene-by-gene basis. A full dataset phylogeny reduces gene specific bias and can support reciprocally monophyletic species if SNPs reflecting that separation are prevalent enough, but the phylogeny can also be influenced by noise from older mutations. Phylogenetic analysis of individual genes provides additional information about the extent and distribution of support for monophyly within the genome, but requires careful consideration of how much information is possessed by each individual locus, and that in this case monophyly is an attribute being measured rather than a result being found.

Phylogenetic inference under the multispecies coalescent model (MSC) (Rannala & Yang 2003) is a relatively new method of inferring evolutionary relationships between species. The MSC models gene-trees evolving under stochastic processes generation-by-generation, given a species tree with estimated values for species-divergence (or coalescent) times (τs) for each node and the effective population size (θs) along both modern and ancestral branches. Then the probabilities of each gene-tree matching the species tree can be combined to get a prior probability for the species tree model. When using the MSC to infer species delimitation of two species, there are two species tree models, one in which the two species are distinct lineages that diverged at time τ and one in which there is only a single unbranching lineage. If the two species represent distinct lineages, we expect to see a high probability for the two-species model, and a low probability for the single-species model. One major drawback of the MSC is that it is very sensitive to the parameter priors (Chan & Grismer 2019), so it is important to check the parameters (particularly θ) empirically against the data.

Another indicator of the presence of evolutionarily distinct lineages is the pattern of genetic divergence within- versus between-groups. P-distance, or pairwise genetic distance, between two sequences is the proportion of nucleotide sites that are different relative to the entire sequence length. This metric is used in genetic barcoding to determine the level of similarity between samples (Hubert et al. 2008) and 'bin' them into distinct clusters. As a result of geneflow within species but not across species, p-distance between individuals of the same lineage will be very low in comparison to that with individuals outside of the lineage. Thus, if two species are valid, the average interspecific distance will be greater than the average intraspecific distance. If there is only a single species, average interspecific and intraspecific distances will be approximately the same. Liu et al. (2017) illustrated how this distance can be more detectable using multilocus data such as the exon-capture method used in this study, especially in cases were distance-based barcoding approaches using traditional mitochondrial markers fail to capture variation on their own. In their study, when two species represent true evolutionary lineages, the average intraspecific p-distance converges on a smaller value than the average interspecific p-distance as more loci are incorporated. In doing so, this approach also provides a rough estimate of the number of loci sufficient to distinguish species at that given level of evolutionary divergence.

An alternative approach to species delimitation involves an analysis of admixture. Admixture analysis is a type of clustering analysis often used in population genetics studies to determine the number of populations within a dataset (K) and the genetic composition of individuals included in each (Pritchard et al. 2000; Patterson et al. 2006). Unlike the previous methods, the admixture analysis determines grouping *a priori*, and therefore is unaffected by species identifications. The analysis also provides a way to assess how many groupings best-fit the data using a cross-entropy statistic. Lastly, this method calculates admixture coefficients for each individual, which has the potential to show patterns of hybridization and misidentification. For a species delimitation analysis of two putative species, if the two species represent distinct evolutionary lineages, we would expect admixture analysis to converge on a model favoring two populations (K=2, instead of K=1 or K>2), where groupings reflect the two species identifications and the admixture coefficients for each individual to strongly favor the grouping for that respective species. If our two putative species are not evolutionarily distinct lineages and instead had a high degree of admixture, then the model would converge on a single population (K=1), or in the case of a K=2 model, each sample would have equal contribution from the two admixture groupings.

The goal of this study is to apply each of the species limitation methods discussed here to our flatfish exon-capture dataset, assess the support for each species pair as being two genetically distinct lineages, and compare the results to the established species descriptions. If our data show support for distinct lineages, we can hypothesize that the morphological characters used to describe these species are taxonomically viable. Conversely, if our data are unable to resolve two distinct species, current species designations may need to be reconsidered.

Methods

Samples

Frozen fin and muscle tissues were collected from the eight species of interest: 7 individuals of *Atheresthes evermanni*, 6 *A. stomias*, 15 *Hippoglossoides elassodon*, 15 *H. robustus*, 7 *Lepidopsetta bilineata*, 5 *L. polyxystra*, 5 *Limanda aspera*, and 6 *L. sakhalinensis*. Individuals were selected from geographically diverse locations throughout each species range to capture higher genetic diversity within that species (Figure 15). Tissues for an additional 38 flatfish species (29 Pleuronectidae and 9 Paralichthyidae) were also sampled to serve as outgroups during phylogenetic analysis. Exon-capture libraries were prepared using the double-capture method from Li et al. (2013). Sequenced reads were then assembled into loci and aligned based on the Assexon pipeline from Yuan et al. (2020).

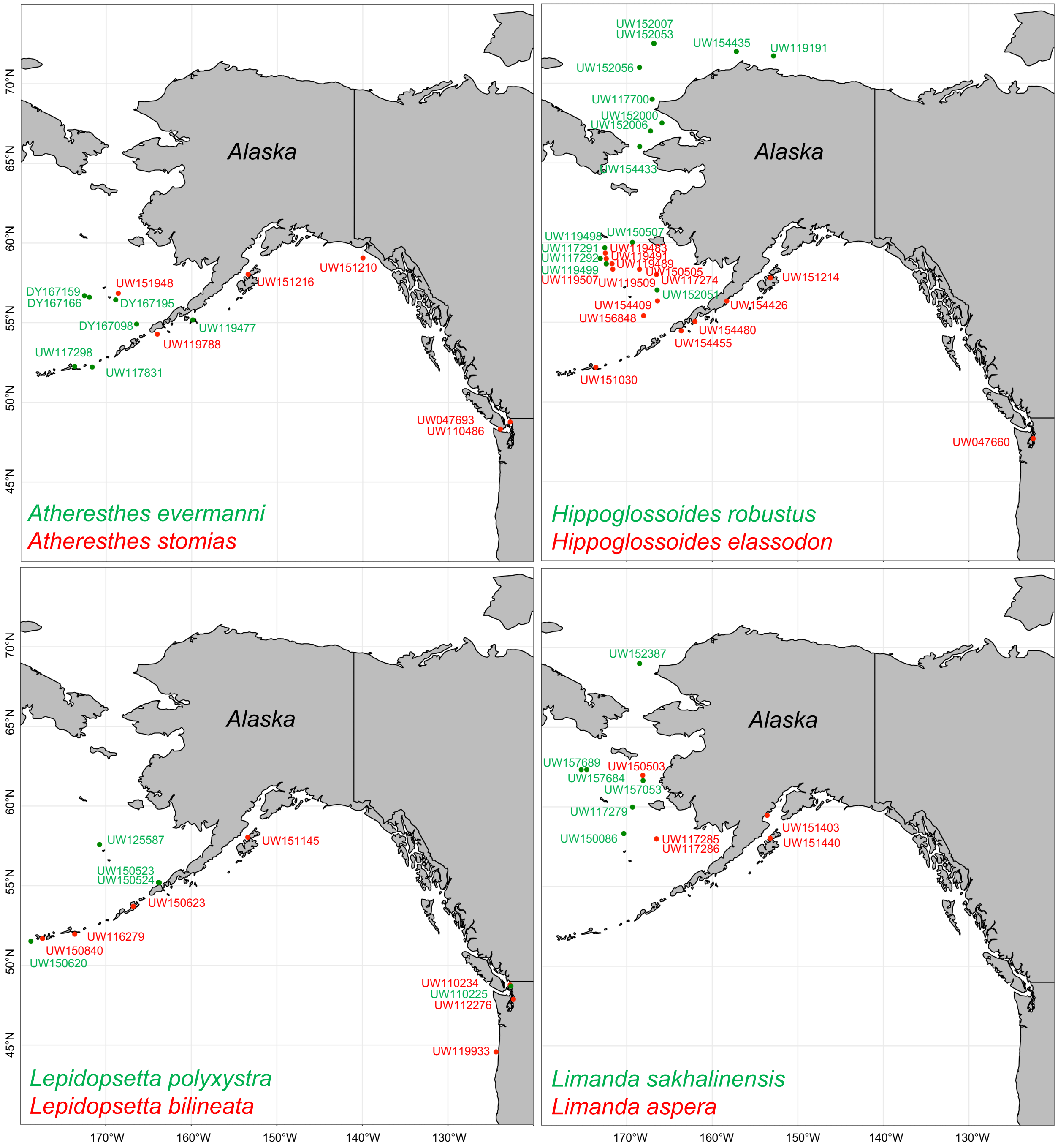


Figure 15: Map of collections sites for eight target species; made with Natural Earth; UW accession numbers listed where applicable; specimen numbers beginning with 'DY' are uncatalogued larvae collected by the NOAA Alaska Fisheries Science Center.

Phylogenetic inference

The aligned dataset for all samples consisted of 3,705 loci. Two tree reconstruction methods were applied to these data. First, we used the concatenation-based method, in which all the loci for a given sample were combined into a single sequence, which was then used to infer a phylogeny in RAxML using the GTRCAT substitution model (Stamatakis 2006, 2014). A second tree was constructed by inferring a gene-tree for each locus under the same conditions as above, then gene-trees were used to collectively infer a species-tree using ASTRAL (Zhang et al. 2018).

We also were interested in assessing how widely reciprocal monophyly of species pairs was supported by individual loci and investigating the properties of the loci that supported monophyly. To do this, support for monophyly of each species and genus was tested in each gene to quantify and identify which genes contain information that supports a division of species. A topology-based test and bootstrap test were conducted with the R package 'spider' (Brown et al. 2012). In the topology-based test, the gene-trees in which a target species was found to be monophyletic were counted. For the bootstrap test, the threshold for monophyletic support was more stringent, only including gene-trees where the monophyletic clade was supported by a bootstrap value of greater than 0.8. In both tests, genes containing data for only one or no individuals within the target group were ignored. To determine whether the genes that supported monophyly of particular species had similar properties. Specifically, we considered gene length, substitution rate (average p-distance), and molecular clocklikeness (MCL) were tested. For each species pair, we compared the values for length, p-distance, and MCL for genes supporting monophyly versus genes that did not, using a two-sample t-test.

Coalescent-based species delimitation

Phylogenetic inference under the MSC was conducted using the program BPP (Yang 2015). For each species pair, sequence data for all non-target species were removed. Species delimitation was inferred for the two species without a guide tree (analysis A11) using theta (θ) prior = invgamma(3, 0.01), and tau (τ) prior = invgamma(3, 0.01). Markov chain Monte Carlo (MCMC) was allowed to run for 10,000 burnin cycles and 50,000 samples. Each analysis tested two species-delimitation models with uniform rooted trees as the species model prior (0.5 for each model). Model '0' represented the single-species hypothesis, and model '1' represented the two-species hypothesis.

Genetic distance between versus within species

A goal of our study was to examine patterns of genetic variation between and within species for our four species pairs, and see what the effect of increasing numbers of loci was on the ability to detect patterns consistent with the presence of distinct species (e.g. low variation within groups, high variation between groups). For each species pair, sequence data for all non-target species were removed and only loci that contained data for all target species individuals were retained. The resulting datasets contained 419 loci for *Atheresthes*, 526, for *Hippoglossoides*, 1065 for *Lepidopsetta*, and 805 for *Limanda*. For a series of selected sample-sizes, genes were randomly selected, concatenated, and p-distance matrices were calculated using the dist.hamming function in the R package 'phangorn' (Schliep 2011). A mean value was calculated for p-distances between individuals of the same species and for p-distances between individuals of different species. Mean values were calculated for 30 random samples of genes at each sample-size.

Admixture analysis

A structure-like analysis was done to identify possible hybrids, which would receive mixed admixture coefficients from both species populations. For each species pair, a structure-like analysis was done on the filtered datasets used for the p-distance analysis. Sample data were treated as haplotype since a single consensus sequence was constructed for each individual. Alleles were determined by finding unique combinations of all variable sites within each gene. There were one to 74 variables sites per gene with a mean of 16.3 across all species pairs. Cross-entropy was calculated for models with one to ten populations to empirically detect genetic groupings. although the two population (K = 2) model was chosen to test our species-pair hypothesis. Admixture coefficients were calculated for each individual using snmf in the R package LEA (Frichot & François 2015) using the K = 2 population model. The results from a K = 3 population model are also provided as an additional figure (Figure S2).

Results

Phylogenetic inference

In both the concatenation-based and species-tree phylogeny (Figure 16), monophyly of all four genera of the species complexes were strongly supported (BS=100; PP=1), but none of the individual species pairs in each genus resolved as reciprocally monophyletic. The only species that was recovered as monophyletic in both trees was *Limanda sakhalinensis*. In *Limanda*, *Lepidopsetta*, and *Hippoglossoides*, there were groupings that superficially appear to represent the species pairs, with only a few individual samples not sorting into their respective species (*Lepidopsetta bilineata* UW150623, UW151145, *L. polyxystra,* UW150620 *Limanda aspera* UW150503, *Hippoglossoides elassodon* UW117274, UW119509, UW151214, UW154455, UW154480, *H. robustus* UW117291, UW117292, UW150251, UW152000).

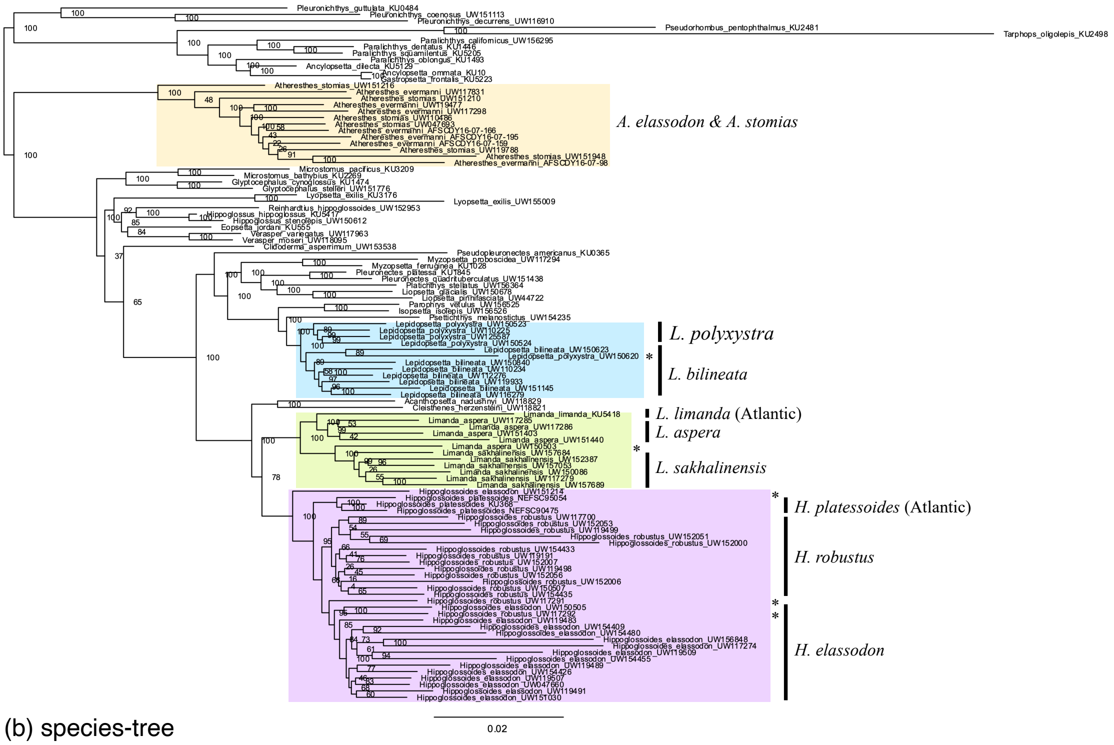
Atheresthes did not sort into any recognizable smaller groups. There was also some uncertainty between the relationships of the *Limanda* and *Hippoglossoides* species pairs and their Atlantic species, with *Limanda limanda* being recovered as sister to *L. aspera*, or more questionably as sister to both other *Limanda* species. *Hippoglossoides platessoides* was recovered as monophyletic but determining its position relative to the other *Hippoglossoides* species was obscured by individuals that failed to group into the major species clusters.

Gene-by-gene monophyly testing

Overall there were few genes (< 8%) that supported the monophyly for tested species (Figure 17). The species of *Lepidopsetta* and *Limanda* were supported by the highest number of genes. Counts for *Hippoglossoides* species had fewer genes passing the ML tree topology test, as there were more individuals sampled and, thus, a higher probability for any one sample to not sort within the clade of its species. There were very few overlapping genes (between 0 and 28) that supported monophyly of both species in a given pair.

Loci that supported monophyly tended to be longer and have a higher average p-distance than loci that did not support monophyly (Table 3). There was no such difference in MCL.

(a) concatenation-based



(b) species-tree

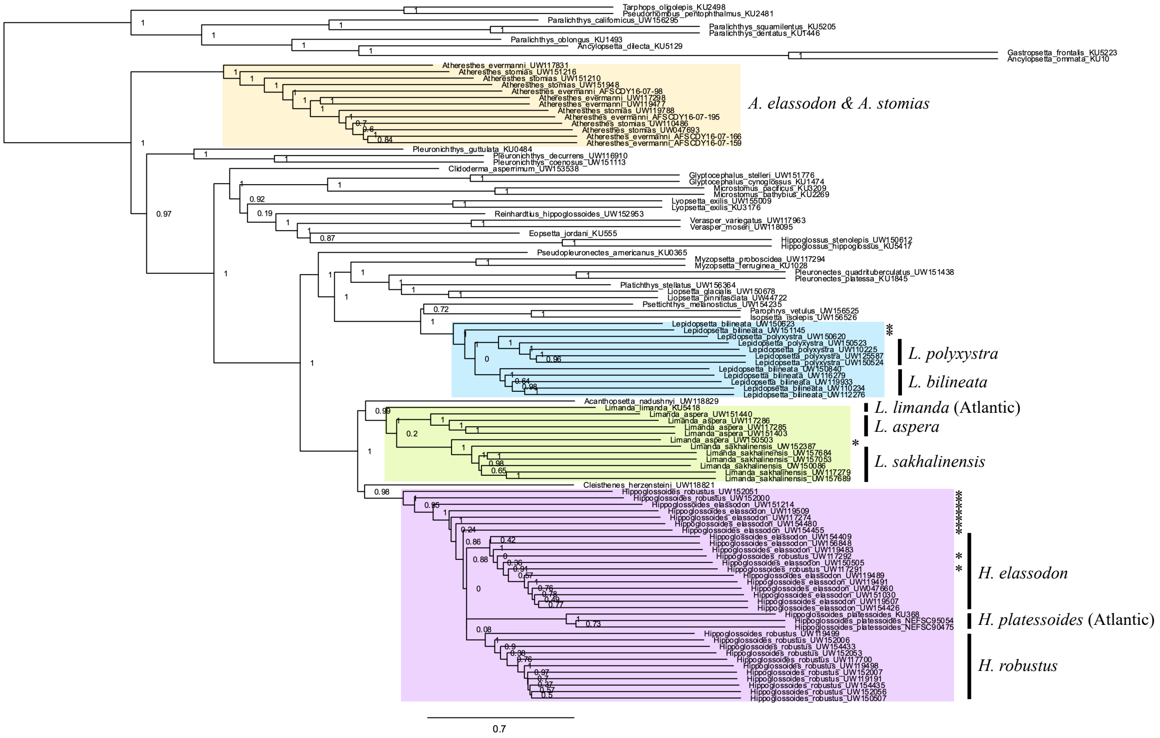


Figure 16: Maximum likelihood phylogenies using the (a) concatenation-based and (b) species-tree method. Nodal support values are shown in (a) bootstrap support and (b) local posterior probability. Clade of each species pair is colored: orange = *Atheresthes*, purple = *Hippoglossoides*, blue = *Lepidopsetta*, green = *Limanda*. Approximate species clades shown using black bars with samples that do not sort into their respective clade represented by an asterisk. There are no clear species clades for *Atheresthes*.

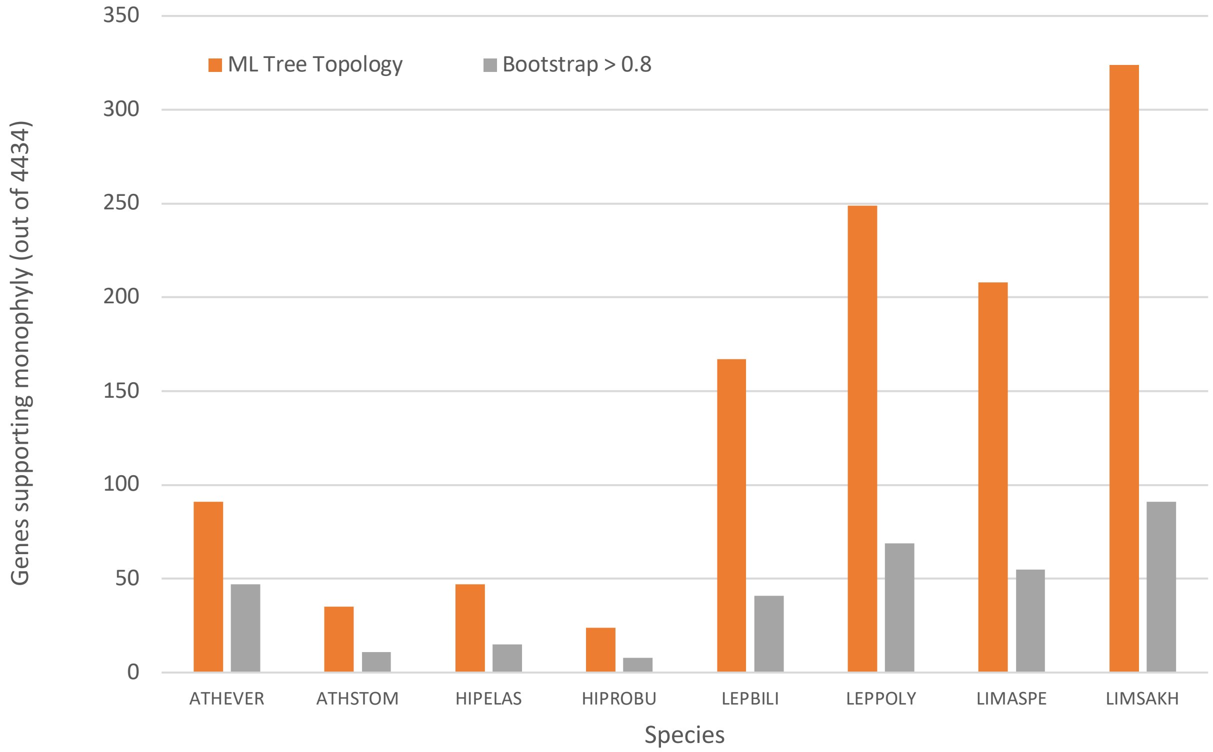


Figure 17: Results from monophyly tests using ML gene tree topology (orange) and bootstrap analysis using 0.8 support as a minimum threshold (gray) for each species. Taxon codes are as follows. ATHEVER = *Atheresthes evermanni*; ATHSTOM = *A. stomias*; HIPELAS = *Hippoglossoides elassodon*; HIPROBU = *H. robustus*; LEPBILI = *Lepidopsetta bilineata*; LEPPOLY = *L. polyxystra*; LIMASPE = *Limanda aspera*; LIMSAKH = *L. sakhalinensis*.

Table 3: Two-sample t-tests of samples with difference variance for length, p-distance, and MCL compared between genes that support monophyly based on the ML tree topology or bootstrap (>0.8) tests and those that do not, using alpha = 0.5. x = genes that support monophyly for either species; y = all other genes (with no monophyly support). Significant p-values < 0.05 are highlighted in red.

	Atheresthes	*Hippoglossoides*	*Lepidopsetta*	*Limanda*
Sample sizes - topology				
size of x =	123	68	375	465
size of y =	4080	4135	3828	3738
Sample sizes - bootstrap (>0.8)				
size of x =	58	23	107	141
size of y =	4145	4180	4096	4062
Test: Length (bp) - topology				
mean of x =	286.32	293.43	294.02	277.53
mean of y =	224.41	225.12	219.58	219.84
p-value =	0.0569	0.0810	< 0.05	< 0.05
Test: Length (bp) - bootstrap (>0.8)				
mean of x	359.12	302.22	283.82	313.98
mean of y	224.36	225.80	224.72	223.17
p-value	< 0.05	0.1893	0.0586	< 0.05
Test: p-distance - topology				
mean of x =	0.0690	0.0719	0.0600	0.0568
mean of y =	0.0567	0.0568	0.0567	0.0571
p-value =	< 0.05	< 0.05	< 0.05	0.8017
Test: p-distance - bootstrap (>0.8)				
mean of x =	0.0755	0.0752	0.0594	0.0561
mean of y =	0.0568	0.0569	0.0570	0.0571
p-value =	< 0.05	< 0.05	0.128	0.5165
Test: MCL - topology				
mean of x =	152.78	203.16	261.59	237.58
mean of y =	248.79	246.69	244.45	247.05
p-value =	< 0.05	0.1801	0.1503	0.2846
Test: MCL - bootstrap (>0.8)				
mean of x =	163.62	208.91	239.41	231.19
mean of y =	247.12	246.20	246.16	246.51
p-value =	< 0.05	0.5357	0.777	0.26

Coalescent-based species delimitation

Based on the reduced datasets containing only target species for each genus, the output of the species delimitation analysis from BPP (Table 4) provided strong support (PP = 1.0) for the two species model in *Hippoglossoides*, *Lepidopsetta*, and *Limanda*. The analysis favored the single species model for *Atheresthes* (PP = 0.93).

Table 4: Posterior probabilities (PP) calculated in BPP analysis A11.

	Atheresthes	***Hippoglossoides***	***Lepidopsetta***	***Limanda***
Model 0 (one species):	0.93	0	0	0
Model 1 (two species):	0.07	1.00	1.00	1.00

Genetic distance between versus within species

As the number of loci sampled from the filtered p-distance datasets increased, the average inter- and intraspecific p-distances for *Atheresthes* and *Hippoglossoides* failed to separate before 400 and 500 sampled loci, respectively (Figure 18). In contrast, the average inter- and intraspecific p-distances for *Lepidopsetta*, and *Limanda* do converge at different values with increasing loci (Figure 19). In both species pairs, the final average interspecific p-distance is marginally greater than intraspecific distance by about 0.002, indicating that their species are genetically dissimilar to one another. This pattern stabilized after about 70 sampled genes in both species pairs.

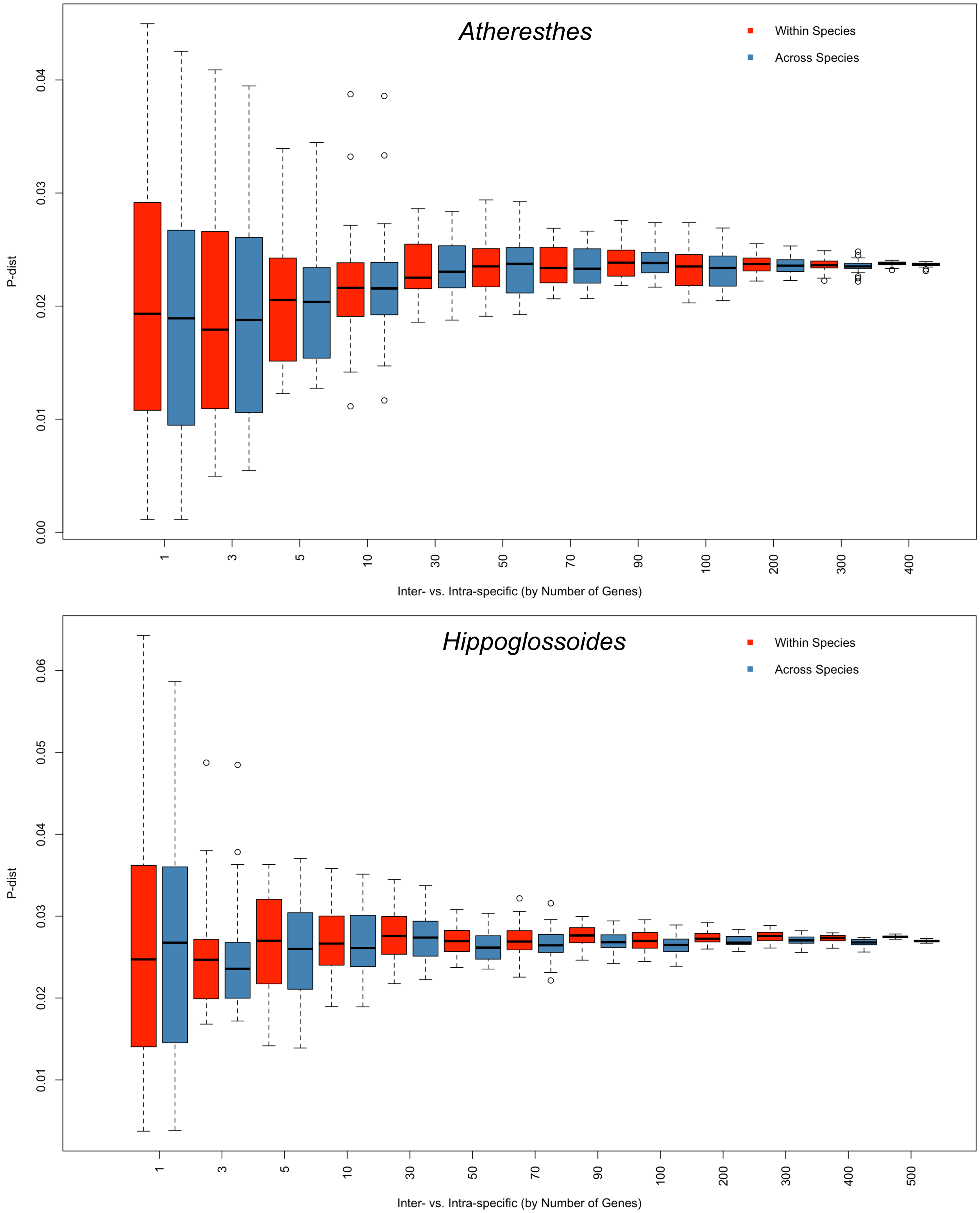


Figure 18: Intra- (red) and interspecific (blue) p-distance of *Atheresthes evermanni* and *A. stomias* (top) and *Hippoglossoides elassodon* and *H. robustus* (bottom) calculated using different number of nuclear loci.

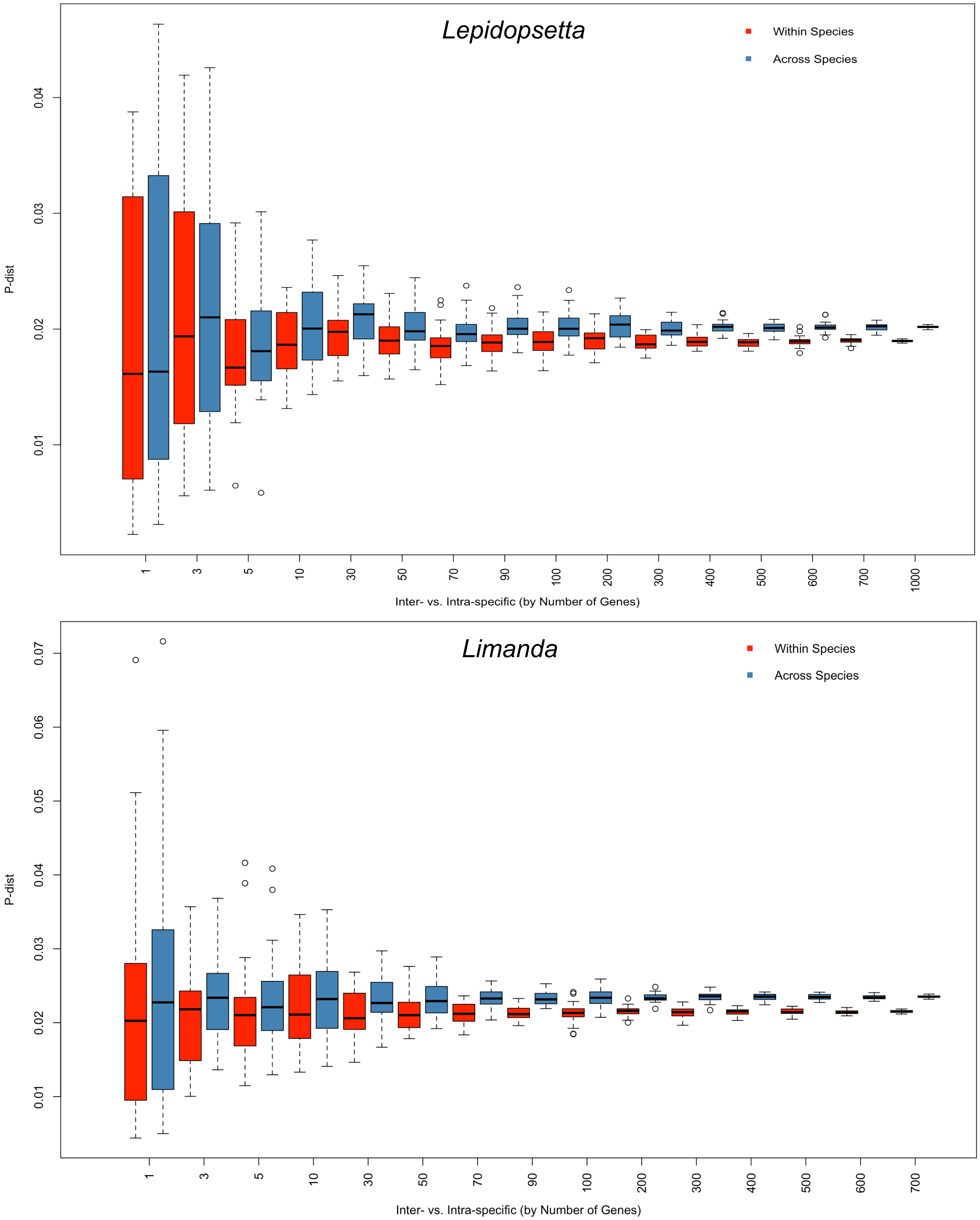


Figure 19: Intra- (red) and interspecific (blue) p-distance of *Lepidopsetta bilineata* and *L. polyxystra* (top) and *Limanda aspera* and *L. sakhalinensis* (bottom) calculated using different number of nuclear loci.

Admixture analysis

Cross-entropy values showed a single population (K = 1) model as the best fit (smallest cross-entropy) for the data across all species pairs (Figure 20). The two population (K = 2) model, which was secondarily favored, was used to calculate admixture coefficients. In *Hippoglossoides*, *Lepidopsetta*, and *Limanda*, the two admixture groups strongly correlated with species pair identifications (Figure 21). In *Hippoglossoides* and *Limanda*, there were a few samples containing a strong admixture component of the other species. *Limanda aspera* UW150503 was more highly associated with the *L. sakhalinen*sis group than the *L. aspera* group. Similarly, there were several *Hippoglossoides* individuals with intermediate admixture contribution from both groups (*Hippoglossoides elassodon* UW154480, *H. robustus* UW117291, 117292). The admixture groupings for *Atheresthes* failed to recreate the groupings based on species identification, however, the red admixture group (Figure 21) was marginally more strongly associated with *A. stomias* (and the green group with *A. evermanni*). Almost all *Atheresthes* individuals were highly associated with one of the admixture groupings.

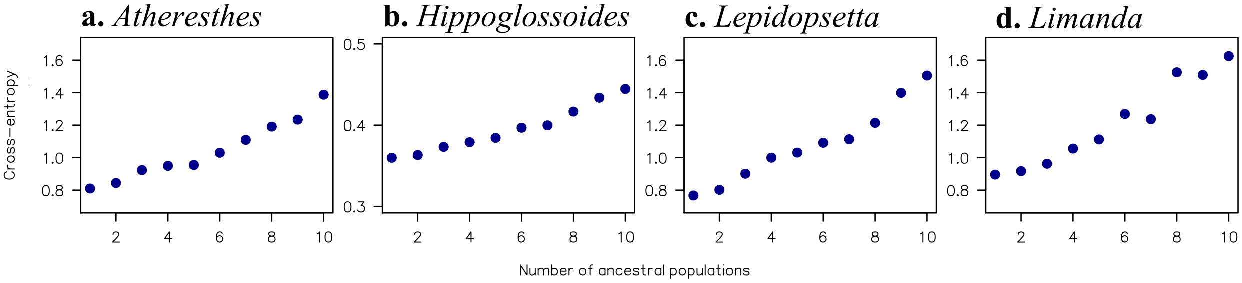


Figure 20: Value of the cross-entropy criterion as a function of the number of populations in snmf for each species pair.

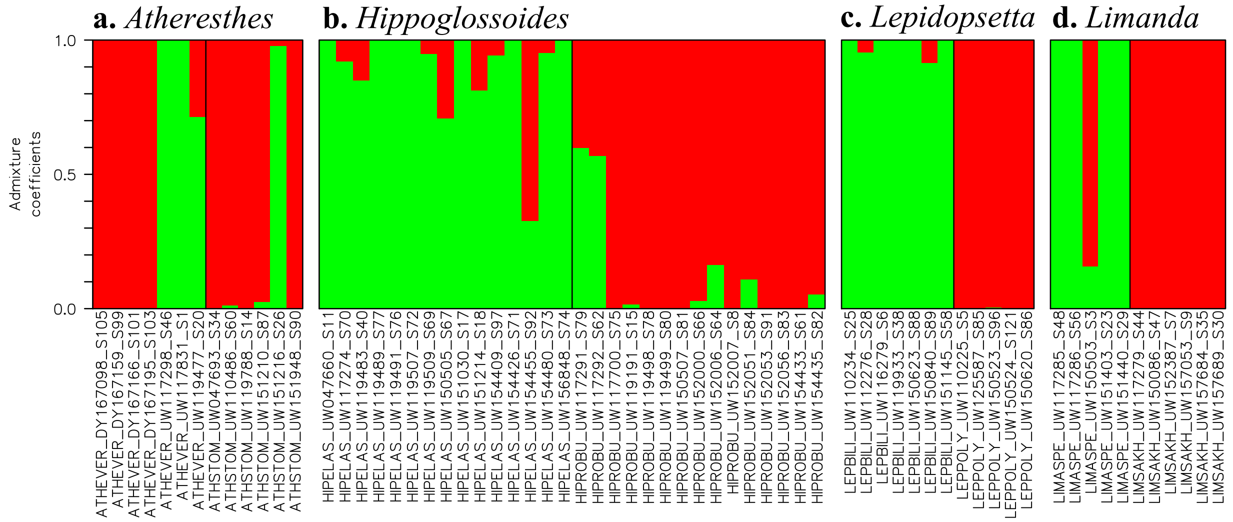


Figure 21: Admixture coefficients for each species pair by individual under a two-population model.

Discussion and Conclusions

Our goal was to use a molecular-based framework for evaluating morphologically defined species as distinct evolutionary lineages. The results from several species delimitation analyses found variable levels of support for each species pair representing two distinct lineages based on exon-capture data (Table 5). There are, however, some largely consistent patterns found across methods. Overall the methods provide evidence for *Lepidopsetta* and *Limanda* species pairs being distinct, with mixed support for *Hippoglossoides* and weak support for *Atheresthes*.

Table 5: Summary of results for each species delimitation analysis. The level of support for there being two distinct lineages for each species pair is listed as strong, moderate, or poor.

	Atheresthes	***Hippoglossoides***	***Lepidopsetta***	***Limanda***
Total evidence phylogeny	poor	moderate	moderate	moderate
Gene-by-gene	poor	poor	moderate	moderate
BPP	poor	strong	strong	strong
p-distance	poor	poor	strong	strong
Admixture	moderate	strong	strong	strong

Before drawing conclusions based on the collective results, we need to consider potential sources of error or bias for each method. Although the total-evidence phylogenetic approach failed to support reciprocal monophyly for any species, this was largely due to a few samples falling outside otherwise well-supported clades. In the gene-by-gene analysis, few genes exhibited monophyly overall, but this was largely due to genes being short and containing little information. While the BPP analysis showed very strong support for two species in *Hippoglossoides*, *Lepidopsetta* and *Limanda*, it should be noted that those data consisted of a single consensus sequence that did not contain information about heterozygosity in individuals. Multiallelic sites contain more variable information that can help identify lineages based on their unique population dynamics, which could result in higher sensitivity to species, and account for patterns of hybridization. The admixture analysis is similarly sensitive to heterozygosity and should also be interpreted with caution.

Atheresthes species showed no pattern of grouping by species in all analyses. Samples were extremely mixed in the phylogenetic analysis, and neither BPP nor p-distance analysis supported there being two distinct lineages, however, the admixture analysis did find some evidence of two genetic groups that were poorly correlated with the species groups. Misidentification or contamination are potential causes for poor sorting. It should be noted that the four *A. evermanni* with specimen numbers starting with ‘DY’ were larval specimens between 10 and 12mm in length. These larvae sorted into the admixture group marginally more associated with *A. stomias* (Figure 21), while the adult *A. evermanni* sorted into the other admixture group. Larval *Atheresthes* can typically be distinguished between 6 and 12mm, and > 18mm based on pigmentation (De Forest et al. 2014). It may be that the timing of pigment development is more variable than what is known, and that these individuals are actually *A. stomias*. *A. stomias* UW151216 was strongly associated with the adult *A. evermanni* admixture group, however this specimen was verified as *A. stomias* based on its morphology and geography, and could be a contaminated sample. Although our data were not able to validate the described species, the existence of two lineages is supported by the diet difference found in these species (Yang & Livingston 1986) and corresponding changes to gill morphology, suggesting a diet played a key role in the speciation process of the two lineages, or was a byproduct thereof. Based on our results, it is likely that the current diagnostic characters are insufficient to reliably sort individuals into distinct lineages, especially for early life stages, and the existence of multiple *Atheresthes* lineages is questionable. In order to better evaluate the possibility of revising *Atheresthes* to contain only one species, a more in-depth population genetics and taxonomic analysis is needed for both larvae and adults of these species using a dense sampling of individuals and morphological characters.

Kartavtsev et al. (2007, 2008a) and Vinnikov et al. (2018) found *Hippoglossoides elassodon* and *H. robustus* to not be reciprocally monophyletic using two and three individuals respectively. Phylogenetic analyses from this study using 15 individuals from each species also showed that reciprocal monophyly of the two species is poorly supported, but there is phylogenetic clustering of individuals with the same species identification. Although p-distance within and between species did not separate for *H. elassodon* and *H. robustus* (Figure 18), both BPP and admixture analysis support current species as distinct genetic lineages, however in the admixture analysis there are several individuals showing admixture, notably *H. elassodon* UW154480, and *H. robustus* UW117291 and 117292, possibly indicating that they are genetic hybrids. Varying admixture ratios can result if fertile hybrids backcross into either species. Individuals with intermediate morphological characters, presumed to be hybrids, are known from this genus, but the questionable species here do not appear to be morphological intermediates based on established diagnostic characters. It is likely that these two *Hippoglossoides* species represent lineages that have relatively high rates of genetic exchange. This can occur if the lineages diverged recently or are still in the process of speciation. The north-south separation in distributions of these species in the Bering Sea may be an indicator of a history of allopatric speciation, with the Bering Strait (above sea-level during periods of glaciation in the Pleistocene) acting as a geographic barrier between two populations (Bering Sea and Beaufort Sea) of an ancestral species. In this scenario genetic mixing can occur after the two populations become reintroduced if the period of separation was insufficient to evolve incompatible reproductive systems. The potential for these species to continue to deviate or remerge into a single lineage depends on many environmental and biological factors (eg. bathymetry, seasonality of ocean chemistry, reproductive biology, migratory behavior, food availability, competition) and the rates at which both change. As such, the outcome is very challenging to predict. Vinnikov et al. (2018) argued that *H. robustus* be a junior synonym of *H. elassodon* (as an Arctic subspecies), but our species delimitation methods did find moderate support for two lineages, and show that they are mostly discernable through morphology. A more in-depth population genetics and taxonomic study using more samples and additional morphological characters would be required to justify a revision to merge the two species.

Lepidopsetta bilineata and *L. polyxystra* were very well supported as distinct lineages by BPP, p-distance, and admixture analyses. The phylogenetic analysis also found good support for two species groups, albeit with one or two samples that did not sort into their respective clade. In their revision of *Lepidopsetta*, Orr & Matarese (2000) identified diagnostic morphological characters for both adults and larvae, and described differences in their spatial distribution, but did not utilize genetic evidence. This study provides a genetic basis to further support their findings, that these species are indeed distinct evolutionary lineages and that the established diagnostic characters reliably sorts individuals into their lineages.

Limanda aspera and *L. sakhalinensis* were strongly supported as distinct lineages in all species delimitation analyses. The specimen *L. aspera* UW150503, which grouped with *L. sakhalinensis* in the phylogenetic and admixture analysis was verified to be *L. aspera* based on morphology. At such contrasting admixture values, it may be that this specimen was contaminated. Both *Limanda* species have long been known with diagnostic traits both preserved and in life, and differ longitudinally in their spatial distribution. The larval morphology of these species were also recently described (Busby et al. 2017). As with *Lepidopsetta* our findings further validate that these *Limanda* species are distinct lineages that can be diagnosed using morphological characters.

In addition to the eight species explored in this study, a vast majority of managed species in Alaska were described entirely based on morphological characters. This study illustrates the potential for these morphologically defined species to not represent distinct evolutionary lineages, and the need to corroborate morphological data with genetic data. In managed species, it is imperative to test morphological species, as effective management of a fishery is dependent on the knowledge of true interbreeding populations and ultimately their reproductive potential. This is particularly crucial in closely related species with similar morphologies, trophic niches, and distributions. In order to robustly establish appropriate species for the purpose of management as recommended for *Atheresthes* and *Hippoglossoides* in this study, a taxonomic analysis should be conducted using a large sample of individuals using both used and novel characters, and including genetic data for all individuals. A priority should first be placed on empirically establishing genetically distinct groups, then identifying synapomorphies and diagnostic traits. Diagnostic morphological characters should be carefully selected to accurately identify individuals accounting for any variation. If there is strong evidence of there being only a single species (with support from many species delimitation methods) then previous morphospecies may describe intraspecific variation and potentially phenotypic plasticity. In cases where there is strong support for multiple lineages that are morphologically indistinguishable, establishing a separate species for each may not be desirable, as it is inefficient and impractical to implement genetic identification for stock assessment purposes. One solution could be to keep a single species designation but consider different populations into predictive models. Ultimately, a species should be defined with the purpose of providing the most utility for effective management.

Delimiting species is a challenging endeavor given the natural continum of divergence seen throughout the process of speciation, but species must be established before understanding further aspects of ecology (abundance, spatial distribution, evolutionary history, life history, interactions with other organisms, risk of extinction or extirpation, etc.). Management entities are dependent on such ecological knowledge to inform decisions that aim to preserve natural resources.

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Additional Figures(s)

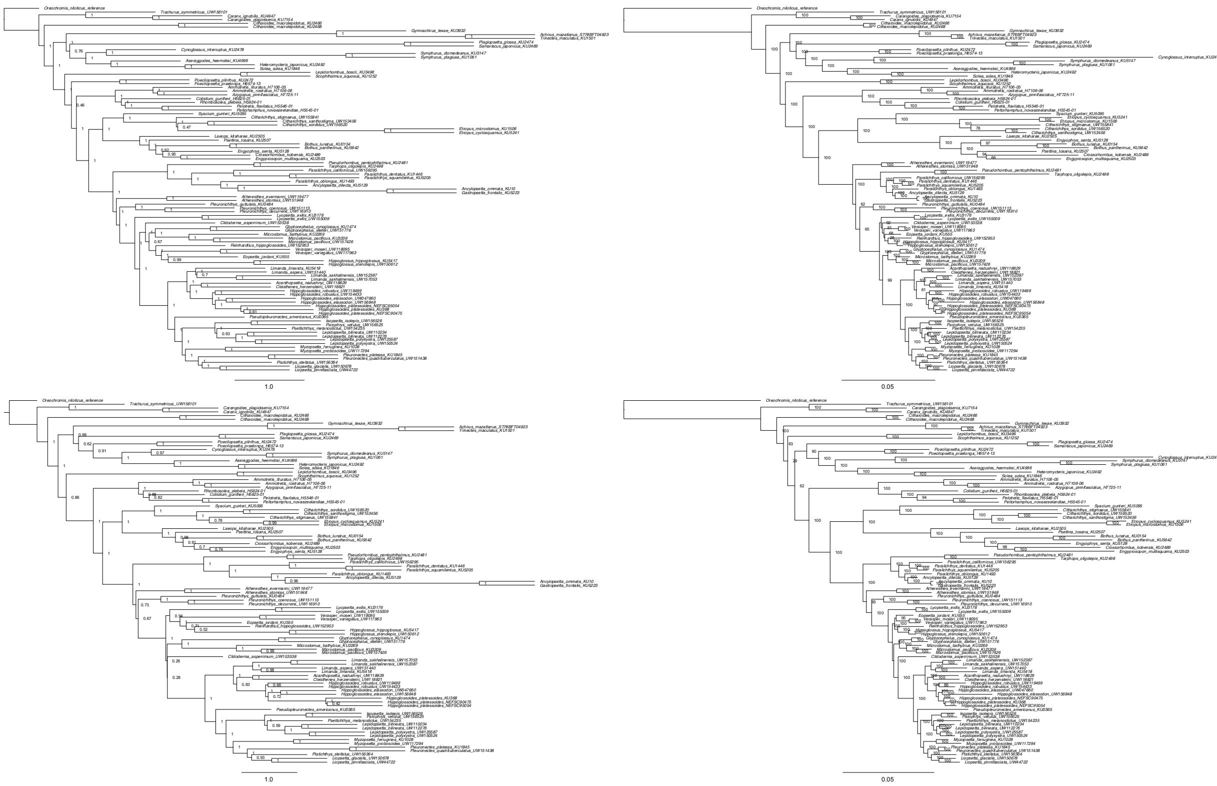

Figure S1: Trees from chapter 1 with all support values shown: top-left = unfiltered-concatenation-based ML, top-right = unfiltered-species-tree, bottom-left = clocklike-concatenation-based ML, bottom-right = unfiltered species-tree

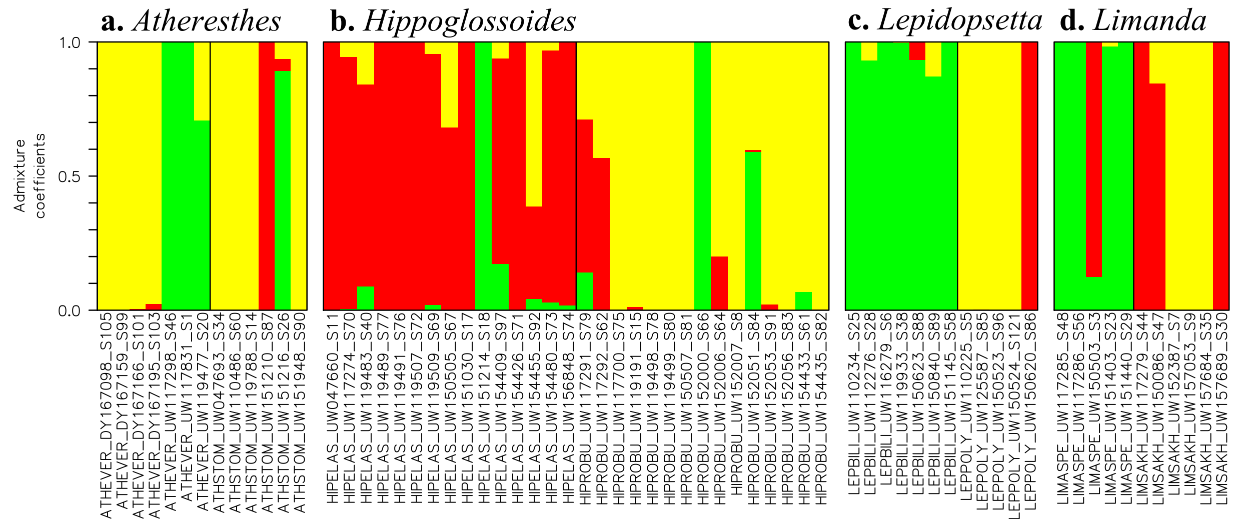

Figure S2: Admixture coefficients for each species pair by individual under a three-population model.