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Multi-locus fossil-calibrated phylogeny of Atheriniformes (Teleostei, Ovalentaria)

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ABSTRACT

Phylogenetic relationships among families within the order Atheriniformes have been difficult to resolve on the basis of morphological evidence. Molecular studies so far have been fragmentary and based on a small number taxa and loci. In this study, we provide a new phylogenetic hypothesis based on sequence data collected for eight molecular markers for a representative sample of 103 atheriniform species, covering 2/3 of the genera in this order. The phylogeny is calibrated with six carefully chosen fossil taxa to provide an explicit timeframe for the diversification of this group. Our results support the subdivision of Atheriniformes into two suborders (Atherinopsoidei and Atherinoidei), the nesting of Notocheirinae within Atherinopsidae, and the monophyly of tribe Menidiini, among others. We propose taxonomic changes for Atherinopsoidei, but a few weakly supported nodes in our phylogeny suggests that further study is necessary to support a revised taxonomy of Atherinoidei. The time-calibrated phylogeny was used to infer ancestral habitat reconstructions to explain the current distribution of marine and freshwater taxa. Based on these results, the current distribution of Atheriniformes is likely due to widespread marine dispersal along the margins of continents, infrequent trans-oceanic dispersal, and repeated invasion of freshwater habitats. This conclusion is supported by post-Gondwanan divergence times among families within the order, and a high probability of a marine ancestral habitat.

1. Introduction

The order Atheriniformes includes about 350 fish species commonly known as silversides, rainbowfishes, and blue eyes, many of which are important to support commercial fisheries and the aquarium trade (Eschmeyer, 2013; Nelson, 2006). They inhabit a wide range of environments from freshwater lakes, lagoons and rivers, to estuaries and coastal marine waters, and are globally distributed in tropical and temperate regions (Table 1). Some atheriniform species are exclusively marine, but many others are restricted to freshwater (Nelson, 2006) and some diadromous species undertake seasonal migrations between marine and freshwater habitats (Dyer and Chernoff, 1996). Many atheriniforms exhibit a wide range of salinity tolerance typical of euryhaline species. The order is moderately diverse in terms of morphology, with adult body sizes ranging from 25 mm to 520 mm in length (Dyer and Chernoff, 1996). Most species are silvery in color with a prominent silvery lateral stripe but rainbowfishes (Fam. Melanotaeniidae) can be very colorful, especially males (Dyer, 1998; Dyer and Chernoff, 1996). The most bizarre morphology among atheriniforms is found in priapium fishes (family Phallostethidae), with male phallostethids exhibiting sub-cephalic copulatory organs derived from modifications of the pelvic skeleton (Parenti, 1986).

The monophyly of Atheriniformes is supported by ten morphological synapomorphies (Dyer and Chernoff, 1996). Monophyly also is supported by molecular analyses of mitogenomes for a small number of taxa (Setiamarga et al., 2008), by analyses of cytochrome *b* and RAG-1 data for 47 ingroup taxa (Bloom et al., 2012), but not by a parsimony analysis combining morphology, mitochondrial, and nuclear gene data (Sparks and Smith, 2004). A recent molecular phylogenetic study of 1416 ray-finned fishes based on 21 gene fragments that included 25 atheriniform taxa from 8 of the 11 recognized families also resolved the monophyly

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Table 1
Families of Atheriniformes according to Nelson (2006) and Eschmeyer and Fong (2014), valid genera, their geographic distribution, habitat type, and alternative taxonomic arrangements.

Family and common name	Geographic distribution and habitat	Included genera	Taxonomic observations
Atherinidae Old World Silversides	Indo-West Pacific and Atlantic; freshwater, marine, and brackish	*Alepidomus, Atherina, Atherinason, Atherinomorus, Bleheratherina*[A], *Craterocephalus, Hypoatherina*[A], *Kestratherina, Leptatherina, Sashatherina*[A], *Stenatherina*[A], *Teramulus*[A]	Number of subfamilies included varies between two[a,b], three[c], six[d], and nine[e]; 12 genera with 68 species
Atherinopsidae New World Silversides	North, Central, and South America (Atlantic and Pacific); freshwater, marine, and brackish	*Atherinella, Atherinops, Atherinopsis, Basilichthys, Chirostoma, Colpichthys*[A], *Labidesthes, Leuresthes, Melanorhinus, Membras, Menidia, Odontesthes, Poblana*	Formerly a subfamily in Atherinidae[a,d,e], later recognized as a family[b]; includes two subfamilies (Atherinopsinae and Menidiinae); 13 genera with 109 species
Atherionidae Pricklenose silversides	Indian Ocean and Western Pacific; marine	*Atherion*	Previously a subfamily of Atherinidae[e,f], later elevated to family[c,g]; 1 genus with three species
Bedotiidae Madagascar rainbowfishes	Central and eastern Madagascar; freshwater	*Bedotia, Rheocles*	Previously a subfamily of Melanotaeniidae[c], later elevated to family[h]; 2 genera with 16 species
Dentatherinidae Tusked silversides	Tropical western Pacific; marine	*Dentatherina merceri*[A]	Formerly a subfamily of Atherinidae, a subfamily of Phallostethidae[i], or as a separate family[j]; monotypic
Isonidae Surf silversides	Indo-West Pacific; marine	*Iso*	Either within Notocheiridae[c], or as its own family[b,k]; five species
Melanotaeniidae Rainbowfishes	Australia, New Guinea, eastern Indonesia; freshwater, few brackish, rarely marine	*Cairnsichthys, Chilatherina, Glossolepis, Iriatherina, Melanotaenia, Pelangia*[A], *Rhadinocentrus*	Also as a subfamily within Melanotaeniidae[c];seven genera with 80 species
Notocheiridae Surf silversides	Southern South America; marine	*Notocheirus hubbsi*	Also as a subfamily in Atherinopsidae[k], sister to Atherinopsidae[g], or with *Iso* in a monophyletic family[c]; monotypic
Phallostethidae Priapium fishes	Southeast Asia (Philippines to Thailand and Sumatra); freshwater and brackish	*Gulaphallus*[A], *Neostethus, Phallostethus*[A], *Phenacostethus*[A]	Proposed as a subfamily within Phallostethidae, sister to Dentatherininae[c,g]; 4 genera with 23 species
Pseudomugilidae Blue eyes	Australia and New Guinea; freshwater and brackish, rare marine	*Kiunga, Pseudomugil, Popondichthys*[A], *Scaturiginichthys*[A]	Formerly a subfamily within Melanotaeniidae[c]; 4 genera with 18 species
Telmatherinidae Sailfin silversides	Sulawesi, Misool and Batanta Island; freshwater	*Kalyptatherina, Marosatherina, Paratherina*[A], *Telmatherina*[A], *Tominanga*[A]	Formerly a subfamily within Melanotaeniidae[c]; 5 genera with 18 species

[a] Fowler (1903).
[b] Saeed et al. (1994).
[c] Dyer and Chernoff (1996).
[d] Jordan and Hubbs (1919).
[e] Schultz (1948).
[f] Patten (1978).
[g] Aarn and Ivantsoff (1997).
[h] Stiassny et al. (2002).
[i] Parenti (1984).
[j] Parenti and Louie (1998).
[k] Bloom et al. (2012).
[A] Genera not sampled in this study.

of this order with high bootstrap support (Betancur et al., 2013). In that study, Atheriniformes was resolved within the same clade (superorder Atherinomorphae) as Beloniformes and Cyprinodontiformes, in agreement with previous hypotheses (Parenti, 1993).

The number of atheriniform families and their composition have been relatively variable over time (Nelson, 2006). A summary of current hypotheses is presented in Table 1. Interrelationships of Atheriniformes have been examined by several authors based on morphological characters (Aarn and Ivantsoff, 1997; Dyer and Chernoff, 1996; Ivantsoff et al., 1987; Parenti, 1984, 1993; Rosen, 1964; Rosen and Parenti, 1981; White et al., 1984) and DNA sequence data (Betancur et al., 2013; Bloom et al., 2012; Setiamarga et al., 2008; Sparks and Smith, 2004). Within the order, extensive disagreement persists among phylogenetic studies (Fig. 1), especially comparing the very different conclusions reached by Dyer and Chernoff (1996) and Aarn and Ivantsoff (1997). The recognition of two suborders, based on cladistics analysis by Dyer and Chernoff (1996), however, is supported by most studies that place the New World silverside family Atherinopsidae (suborder Atherinopsoidei) as the sister-group to the remaining families (Fig. 1A, B, D, E, F). Within Atherinopsoidei, recent molecular analyses (Bloom et al., 2012) supported previous hypotheses by Saeed et al. (1994) and Aarn and Ivantsoff (1997) regarding the composition of the family Notocheiridae (“surf silversides”). These authors placed *Notocheirus hubbsi*, the surf silverside from temperate coastal waters of Argentina and Chile, closer to or nested within the family Atherinopsidae (Fig. 1B and D). A second genus of surf silversides (*Iso*, with five Indo-Pacific species) traditionally included in the family Notocheiridae was shown to be distantly related and assigned to Isonidae, suggesting that many of the morphological characters that were used to support the monophyly of Notocheiridae (*Iso* + *Notocheirus*) may be convergent (Bloom et al., 2012). The rest of the families are distributed in the Old World (Table 1) and included in the suborder Atherinoidei (Dyer and Chernoff, 1996; Nelson, 2006). However, this early phylogenetic split between New World and Old World lineages was not obtained by Sparks and Smith (2004), who placed Atherionidae and Phallostethidae among outgroup taxa that included species

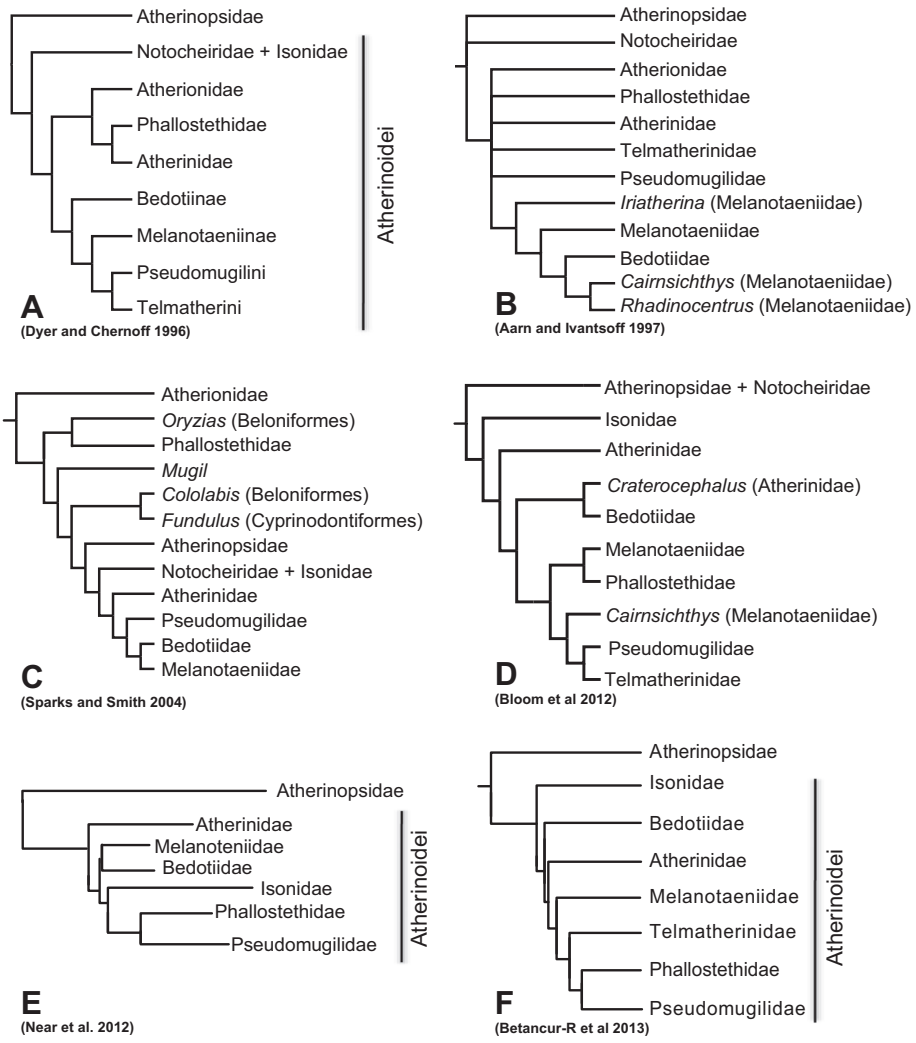

Fig. 1. Phylogenetic hypotheses for families in the order Atheriniformes based on previous studies. A: Dyer and Chernoff (1996); B: Aarn and Ivantsoff (1997); C: Sparks and Smith (2004); D: Bloom et al. (2012); E: Near et al. (2012); F: Betancur-R et al. (2013).

from the orders Beloniformes, Cyprinodontiformes, and Mugiliformes, challenging the monophyly of Atheriniformes. Extensive disagreement exists among previous hypotheses, for example in relation to the position of the morphologically peculiar Phallostethidae, the monophyly of Melanotaeniidae, and relationships among Indo-West Pacific families Melanotaeniidae, Pseudomugilidae, Bedotiidae, and Telmatherinidae (Aarn and Ivantsoff, 1997; Aarn et al., 1998; Dyer and Chernoff, 1996; Ivantsoff et al., 1997; Saeed et al., 1994; Sparks and Smith, 2004).

Relationships within atheriniform families have been studied using morphology (Aarn and Ivantsoff, 1997; Aarn et al., 1998; Dyer, 1997; Parenti, 1993; Saeed et al., 1994), molecular markers (Bloom et al., 2009; McGuigan et al., 2000; Unmack et al., 2013; Zhu et al., 1994) or combined datasets (Dyer, 1997; McGuigan et al., 2000; Sparks and Smith, 2004). A consensus for the phylogenetic relationships for most families is yet to emerge.

The oldest Atheriniform fossils are marine/brackish species of the extinct genus *Hemitrichas* Peters 1877 (Atherinidae) from the Miocene and Oligocene in Germany, Switzerland, and Iran (Gaudant and Reichenbachen, 2005; Jost et al., 2007; Malz, 1978; Reichenbacher, 2000; Reichenbacher and Weidmann, 1992; Weiler, 1942; Weiler and Schäfer, 1963). The few molecular phylogenies that attempted to estimate divergence dates for Atheriniformes reported highly discordant results. A comprehensive phylogenetic study of bony fishes that used 60 fossil calibrations placed the origin of this order around 70 million years ago (Ma) (Betancur et al., 2013), but other studies focusing specifically on the age and distribution of one or a few families estimated significantly older ages (Unmack and Dowling, 2010). Uncertainty about divergence dates for atheriniforms and their distribution in marine and freshwater environments inspired a diversity of biogeographic scenarios to explain their cosmopolitan distribution. Vicariance hypotheses based on the fragmentation of continental masses, however, can be explicitly tested with time-calibrated phylogenies (Crisp et al., 2011). If the origin of most atheriniform families is Cenozoic, by which time the continents already occupied their modern location (Upchurch, 2008), their current distribution must have been affected mostly by dispersal rather than by vicariance. Marine to freshwater transitions have occurred in several Atheriniform lineages (Beheregaray, 2000; Bloom et al., 2013; Pujolar et al., 2012), probably facilitated by historical changes in global sea levels that have led to the colonization of coastal freshwater environments by marine ancestors. Examples of these speciation events in Atheriniformes are the *Atherina boyeri* complex in Europe (Pujolar et al., 2012), the inland silverside *Menidia beryllina* (Fluker et al., 2011) in North America, and *Odontesthes* in Southern Brazil (Beheregaray, 2000; Beheregaray et al., 2002). A robust phylogenetic hypothesis is a

necessary framework to interpret the evolution of habitat preference in such widespread species (Betancur-R et al., 2012). The aims of this study are to provide a multi-locus, fossil-calibrated phylogenetic hypothesis for Atheriniformes to establish a solid framework for evolutionary studies, to inform taxonomic decisions, and to test previous biogeographic hypotheses.

2. Materials and methods

2.1. Taxonomic sampling, DNA extraction, PCR, and sequencing

We collected a comprehensive taxon sample that includes 103 species, representing 35 (out of 51) genera, and 10 of 11 families of Atheriniformes – only missing the monotypic family Dentatherinidae (Table 1). Two specimens were used for DNA analysis for most species for quality control; all tissues were already available from museums or from the author's collections. Appendix 1 contains collection locality and museum voucher information (when available). For each family, the number of genera represented in our sample (out of the total number of genera in the family, see Table 1) is as follows: Atherinidae (8 out of 13), Atherionidae (1 out of 1), Atherinopsidae (12 out of 13), Bedotidae (2 out of 2), Isonidae (1 out of 1), Melanotaenidae (6 out of 7), Notocheiridae (1 out of 1), Phallostethidae (1 out of 4), Pseudomugilidae (2 out of 4) and Telmatherinidae (2 out of 5). Five species of Beloniformes and Cyprinodontiformes were used as outgroup. A complete list of taxa used in this study also is provided in Table 2.

Extraction of genomic DNA was performed using DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA). The molecular markers chosen for this study included seven nuclear genes commonly used for fish phylogenetic studies (Betancur et al., 2013): *ficd* (FIC domain-containing protein 334648), *gcs1* (glucosidase 1 or mannosyl-oligosaccharide glucosidase LOC567913), *kbtbd4* (Kelch repeat and BTB (POZ) domain containing 4 LOC393178), *kiaa-l* (leucine-rich repeat and WD repeat-containing protein, KIAA1239-like LOC562320), *myh6* (cardiac muscle myosin heavy chain 6 alpha), *sh3px3* (SH3 & PX domain-containing 3-like protein), and *slc10a3* (solute carrier family 10, member 3; zgc:85947). Nested-PCR amplifications were performed following published protocols for each marker (Betancur-R et al., 2013b; Li et al., 2011, 2008, 2007). In addition to the nuclear genes, the entire mitochondrial cytochrome *b* gene (cytb) was amplified and sequenced as described previously (Lewallen et al., 2011; Unmack and Dowling, 2010). The resulting amplicons were sent for purification and sequencing from both directions to High Throughput Sequencing Solutions (HTSeq.org), University of Washington, Seattle, Washington. Sequences were edited and aligned using Geneious v6 (created by Biomatters, http://www.geneious.com/).

2.2. Phylogenetic analysis

Multiple sequence alignments were performed for each marker separately using MAFFT with default settings (Katoh et al., 2002). After visual inspection to verify expected open reading frames and trimming of sequence ends, individual gene trees were generated with RAxML (Stamatakis, 2006; Stamatakis et al., 2008). Gene trees included duplicate sequences for each species for quality control, to verify the clustering of samples from the same species, and to check for cross-contamination. Sequences that clustered in unexpected positions of the gene tree and did not group with the corresponding duplicate sequence for the same species were re-extracted, re-amplified, and sequenced again. After all sequences passed this quality-control step, a single sequence per species (usually the longest sequence) was used for subsequent analyses. MEGA 5 (Tamura et al., 2011) and Geneious v6 were used to calculate pairwise distances, overall mean distances per marker, percent of invariant sites and pairwise percent identity. Individual gene datasets were concatenated using Geneious v6 (created by Biomatters, http://www.geneious.com/). A first optimal partitioning scheme was obtained with PartitionFinder (Lanfear et al., 2012), starting with 24 *a priori* defined partitions (by gene and codon position), using a greedy algorithm based only on the subset of models of substitution available in MrBayes. Bayesian analysis was performed using MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003), with four independent runs, for 150 million generations each. Maximum likelihood analyses of the partitioned data set (25 replicates) were implemented with RAxML on the CiPRES Science Gateway XSEDE server (Miller et al., 2010), and confidence on the resulting tree was assessed with rapid bootstrapping. A second partitioning scheme was obtained by running PartitionFinder's greedy algorithm under all available models. This partitioning scheme was fully specified for 12 independent runs using Garli 2.0 (Zwickl, 2006, 2011), and BEAST 1.8.0 (Drummond et al., 2012).

A time-calibrated phylogenetic hypothesis was inferred under a Bayesian framework using BEAST 1.8.0. The concatenated dataset was analyzed under an uncorrelated log-normal clock model (UCLN) with seven calibration priors (see below). To account for extinction, we used a birth–death model with an initial mean growth rate of 1, and a relative death rate of 0.1. Phylogenetic placement of calibration points is shown in Fig. 3 (numbered 1–7 on the tree). The placements and prior settings were supported by evidence outlined below.

Calibration 1: the root node for Atheriniformes was defined as a secondary calibration based on a published time-tree for ray-finned fishes inferred for 202 taxa with 59 fossil calibrations (Betancur-R et al., 2013a). Absolute age estimate: 70.5 Ma; 95% soft upper bound: 77.5 Ma, based on the estimated age of Atherinomorpha. Prior setting: normal distribution, Mean = 70.5 Std. Dev. = 4.25.

Calibration 2: Atherinidae (new crown calibration). MRCA: *Craterocephalus stramineus*, *Alepidomus stipes*. Hard minimum age: 23 Ma, †*Hemitrichas stapfi* (Gaudant and Reichenbachen, 2005). Diagnosis and phylogenetic placement: †*H. stapfi* was described as a new species in the family Atherinidae on the basis of its high vertebral number (33–34), high number of abdominal vertebrae (15–16), and otolith morphology with elongated shape, pointed posterior end, and slender and rather long rostrum. Stratigraphic horizon and locality: Quarry "Am Katzenrech", near Dexheim, Germany. Absolute age estimate: Late Oligocene, 23 Ma, based on biostratigraphic dating of the Mainz basin. 95% soft upper bound 70.5 Ma (secondary calibration based on age of Atheriniformes, see calibration 1 above). Prior setting: exponential distribution, Mean = 15.86.

Calibration 3: genus *Atherina* (new crown calibration). MRCA: *Atherina presbyter*, *Atherina boyeri*. Hard minimum age: 10 Ma † *Atherina atropatiensis* (Carnevale et al., 2011). Diagnosis and phylogenetic placement: †*A. atropatiensis* has been included in *Atherina* by the presence of a maxillary ventral shelf, preopercular and infraorbital sensory canals disconnected, dorsally directed anterior palatine process absent, and dorsolateral process of the basipterygium oriented posterodorsally. Comparative analysis with extant *Atherina* show that the meristic characters of † *Atherina atropatiensis* most closely resemble those of *Atherina boyeri*, in addition to simple and non-enlarged haemal arches and spines (Carnevale et al., 2011). These characteristics were used to place this fossil in crown group *Atherina*. Stratigraphic horizon and locality: Lignite beds of the Tabriz Basin, NW Iran. Absolute age estimate: 10 Ma, based on fission track dating of the lignite beds (Reichenbacher et al., 2011); 95% soft upper bound: 23 Ma

Table 2
Taxa and Genbank accession numbers for eight markers sequenced for this study. (n/a = not available).

Family	Genus/species	cytb	ficd	gcs1	kbtbd4	kiaa-l	myh6	sh3px3	slc10a3
Atherinidae	*Alepidomus evermanni*	n/a	KM400820	KM400737	KM400915	KM401014	KM401113	KM401215	KM401313
	Atherina boyeri	EU036422	KM400821	KM400738	KM400916	KM401015	KM401114	KM401216	KM401314
	Atherina breviceps	n/a	n/a	KM400739	KM400917	KM401016	KM401115	KM401217	n/a
	Atherina hepsetus	n/a	n/a	n/a	KM401010	n/a	n/a	KM401308	n/a
	Atherina presbyter	EF439188	KM400822	KM400740	KM400918	KM401017	KM401116	KM401218	KM401315
	Atherinason hepsetoides	KM400684	n/a	n/a	KM400919	KM401018	KM401117	KM401219	n/a
	Atherinomorus lacunosus	KM400687	KM400837	KM400756	n/a	KM401034	KM401134	KM401236	KM401332
	Atherinomorus stipes	JQ282023	KM400839	KM400758	KM400936	KM401036	KM401136	KM401238	KM401334
	Atherinomorus vaigiensis	KM400688	KM400838	KM400757	n/a	KM401035	KM401135	KM401237	KM401333
	Atherinosoma microstoma	KM400685	KM400842	n/a	KM400939	KM401039	KM401139	KM401241	n/a
	Craterocephalus capreoli	GU932792	KM400860	n/a	KM400956	KM401056	KM401157	KM401258	n/a
	Craterocephalus eyresii	GU932886	n/a	KM400772	KM400957	KM401057	KM401158	KM401259	n/a
	Craterocephalus honoriae	GU932765	KM400861	KM400773	KM400958	KM401058	KM401159	KM401260	n/a
	Craterocephalus stercusmuscarum	KM400689	KM400862	n/a	KM400959	KM401059	KM401160	KM401261	n/a
	Craterocephalus stramineus	GU932804	KM400863	KM400774	KM400960	KM401060	KM401161	KM401262	n/a
	Kestratherina esox	GU932762	KM400868	KM400779	KM400965	KM401065	KM401166	KM401266	n/a
	Leptatherina presbyteroides	KM400686	KM400870	n/a	KM400968	KM401068	KM401169	KM401269	n/a
Atherinopsidae	*Atherinella argentea*	JQ282017	KM400823	KM400741	KM400920	KM401019	KM401118	KM401220	KM401316
	Atherinella balsana	KC736414	n/a	KM400742	KM400921	KM401020	KM401119	KM401221	KM401317
	Atherinella blackburni	KC736357	KM400824	KM400743	KM400922	n/a	KM401120	KM401222	KM401318
	Atherinella brasiliensis	KC736412	KM400825	KM400744	KM400923	KM401021	KM401121	KM401223	KM401319
	Atherinella crystallina	KC736346	KM400826	KM400745	KM400924	KM401022	KM401122	KM401224	KM401320
	Atherinella guatemalensis	KC736386	KM400827	KM400746	KM400925	KM401023	KM401123	KM401225	KM401321
	Atherinella hubbsi	KC736388	KM400828	KM400747	KM400926	KM401024	KM401124	KM401226	KM401322
	Atherinella marvelae	JQ282021	KM400829	KM400748	KM400927	KM401025	KM401125	KM401227	KM401323
	Atherinella milleri	KC736379	KM400830	KM400749	KM400928	KM401026	KM401126	KM401228	KM401324
	Atherinella panamensis	KC736362	n/a	KM400750	KM400929	KM401027	KM401127	KM401229	KM401325
	Atherinella pellosemeion	KC736349	KM400831	KM400751	KM400930	KM401028	KM401128	KM401230	KM401326
	Atherinella sallei	KC736383	KM400832	KM400752	KM400931	KM401029	KM401129	KM401231	KM401327
	Atherinella sardina	KC736389	KM400833	KM400753	KM400932	KM401030	KM401130	KM401232	KM401328
	Atherinella schultzi	KC736377	KM400834	n/a	KM400933	KM401031	KM401131	KM401233	KM401329
	Atherinella serrivomer	KC736358	KM400835	KM400754	KM400934	KM401032	KM401132	KM401234	KM401330
	Atherinella starksi	KC736352	KM400836	KM400755	KM400935	KM401033	KM401133	KM401235	KM401331
	Atherinops affinis	KM400705	KM400840	KM400759	KM400937	KM401037	KM401137	KM401239	KM401335
	Atherinopsis californiensis	JQ282018	KM400841	KM400760	KM400938	KM401038	KM401138	KM401240	KM401336
	Basilichthys australis	KM400706	KM400845	KM400763	KM400942	KM401041	KM401142	KM401244	KM401338
	Basilichthys microlepidotus	KM400707	KM400846	KM400764	KM400943	KM401042	KM401143	KM401245	KM401339
	Basilichthys semotilus	JQ282024	KM400847	KM400765	KM400944	KM401043	KM401144	KM401246	KM401340
	Chirostoma attenuatum	KC736405	KM400854	n/a	KM400951	KM401050	KM401151	n/a	KM401347
	Chirostoma consocium	KC736401	KM400855	n/a	KM400952	KM401051	KM401152	KM401253	KM401348
	Chirostoma humboldtianum	KC736402	KM400856	n/a	KM400953	KM401052	KM401153	KM401254	KM401349
	Chirostoma jordani	JQ282072	KM400857	n/a	n/a	KM401053	KM401154	KM401255	KM401350
	Chirostoma labarcae	KC736399	KM400858	KM400770	KM400954	KM401054	KM401155	KM401256	KM401351
	Chirostoma riojai	KC736398	KM400859	KM400771	KM400955	KM401055	KM401156	KM401257	KM401352
	Labidesthes sicculus	JQ282031	KM575708	KM400781	KM400967	KM401067	KM401168	KM401268	KM401357
	Leuresthes tenuis	JQ282032	KM400871	KM400782	KM400969	KM401069	KM401170	KM401270	n/a
	Melanorhinus microps	KC736344	KM400873	KM400784	KM400971	KM401071	KM401172	KM401272	KM401359
	Membras gilberti	JQ282034	KM400883	n/a	KM400981	KM401081	KM401182	KM401282	KM401369
	Membras martinica	JQ282035	KM400884	n/a	KM400982	KM401082	KM401183	KM401283	KM401370
	Menidia beryllina	KC736408	KM400885	KM400791	KM400983	KM401083	KM401184	KM401284	KM401371
	Menidia colei	KC736373	KM400886	KM400792	KM400984	KM401084	KM401185	KM401285	KM401372
	Menidia extensa	KC736370	KM400887	KM400793	KM400985	KM401085	KM401186	KM401286	KM401373
	Menidia menidia	JQ282036	KM400888	KM400794	KM400986	KM401086	KM401187	KM401287	KM401374
	Menidia peninsulae	KC736345	KM400889	KM400795	KM400987	KM401087	KM401188	KM401288	KM401375
	Odontesthes argentinensis	KM400708	KM400893	KM400798	KM400991	KM401090	KM401192	KM401291	n/a

Table 2 (*continued*)

Family	Genus/species	cytb	ficd	gcs1	kbtbd4	kiaa-l	myh6	sh3px3	slc10a3
	Odontesthes bonariensis	KM400709	KM400894	KM400799	KM400992	KM401091	KM401193	KM401292	KM401378
	Odontesthes brevianalis	KM400713	KM400895	KM400800	KM400993	KM401092	KM401194	KM401293	KM401379
	Odontesthes gracilis	KM400714	KM400896	KM400801	KM400994	KM401093	KM401195	KM401294	KM401380
	Odontesthes hatcheri	KM400717	KM400897	KM400802	KM400995	KM401094	KM401196	KM401295	KM401381
	Odontesthes humensis	KM400712	KM400913	KM400817	KM401011	KM401110	n/a	KM401309	KM401396
	Odontesthes incisa	KM400720	n/a	KM400818	KM401012	n/a	n/a	KM401310	KM401397
	Odontesthes ledae	KM400710	KM400898	KM400803	KM400996	KM401095	KM401197	KM401296	KM401382
	Odontesthes mauleanum	KM400716	KM400899	KM400804	KM400997	KM401096	KM401198	KM401297	KM401383
	Odontesthes nigricans	KM400719	KM400900	KM400805	KM400998	KM401097	KM401199	KM401298	KM401384
	Odontesthes perugiae	KM400711	KM400901	KM400806	KM400999	KM401098	KM401200	KM401299	KM401385
	Odontesthes regia	KM400715	KM400902	KM400807	KM401000	KM401099	KM401201	KM401300	KM401386
	Odontesthes retropinnis	n/a	KM400903	KM400808	KM401001	n/a	KM401202	KM401301	KM401387
	Odontesthes smitti	KM400718	KM400904	KM400809	KM401002	KM401100	KM401203	KM401302	KM401388
	Poblana alchichica	KC736395	KM400906	KM400811	KM401004	KM401102	KM401205	KM401304	KM401390
	Poblana ferdebueni	KC736394	KM400907	KM400812	KM401005	KM401103	KM401206	KM401305	KM401391
Atherionidae	*Atherion elymus*	n/a	KM400843	KM400761	KM400940	n/a	KM401140	KM401242	n/a
Bedotiidae	*Bedotia leucopteron*	KM400721	KM400848	n/a	KM400945	KM401044	KM401145	KM401247	KM401341
	Bedotia marojejy	KM400722	KM400849	KM400766	KM400946	KM401045	KM401146	KM401248	KM401342
	Bedotia sp. *Ankavia*	KC133643	KM400850	KM400767	KM400947	KM401046	KM401147	KM401249	KM401343
	Bedotia sp. *Namorona*	KM400734	KM400851	KM400768	KM400948	KM401047	KM401148	KM401250	KM401344
	Rheocles vatosoa	KM400723	KM400911	n/a	KM401009	KM401109	KM401213	KM401307	n/a
	Rheocles wrightae	KC133646	n/a	n/a	n/a	n/a	JX189633	JX189540	n/a
Isonidae	*Iso natalensis*	KM400690	KM400866	KM400777	KM400963	KM401063	KM401164	n/a	n/a
	Iso sp.	JQ282011	n/a	n/a	n/a	KM401111	n/a	KM401311	KM401398
Melanotaeniidae	*Cairnsichthys rhombosomoides*	JQ282005	KM400852	KM400769	KM400949	KM401048	KM401149	KM401251	KM401345
	Chilatherina fasciata	KC133596	KM400853	n/a	KM400950	KM401049	KM401150	KM401252	KM401346
	Glossolepis incisus	GU932788	KM400864	KM400775	KM400961	KM401061	KM401162	KM401263	KM401353
	Iriatherina werneri	JQ282006	KM400865	KM400776	KM400962	KM401062	KM401163	KM401264	KM401354
	Melanotaenia affinis	KM400726	KM400874	n/a	KM400972	KM401072	KM401173	KM401273	KM401360
	Melanotaenia australis	JQ282007	KM400875	KM400785	KM400973	KM401073	KM401174	KM401274	KM401361
	Melanotaenia catherinae	KM400730	KM400876	KM400786	KM400974	KM401074	KM401175	KM401275	KM401362
	Melanotaenia exquisita	KM400727	KM400877	KM400787	KM400975	KM401075	KM401176	KM401276	KM401363
	Melanotaenia kokasensis	KM400731	KM400878	n/a	KM400976	KM401076	KM401177	KM401277	KM401364
	Melanotaenia maccullochi	KM400729	KM400879	KM400788	KM400977	KM401077	KM401178	KM401278	KM401365
	Melanotaenia nigrans	KM400728	KM400880	KM400789	KM400978	KM401078	KM401179	KM401279	KM401366
	Melanotaenia splendida	KC133543	KM400881	n/a	KM400979	KM401079	KM401180	KM401280	KM401367
	Melanotaenia trifasciata	KM400790	KM400882	KM400790	KM400980	KM401080	KM401181	KM401281	KM401368
	Rhadinocentrus ornatus	JQ282009	n/a	KM400816	n/a	KM401108	KM401212	n/a	KM401395
Notocheiridae	*Notocheirus hubbsi*	JQ282012	KM400892	KM400797	KM400990	KM401089	KM401191	KM401290	n/a
Phallostethidae	*Neostethus bicornis*	n/a	KM400890	n/a	KM400988	n/a	KM401189	n/a	KM401376
	Neostethus lankesteri	KM400735	KM400891	KM400796	KM400989	KM401088	KM401190	KM401289	KM401377
Pseudomugilidae	*Kiunga ballochi*	KM400724	KM400869	KM400780	KM400966	KM401066	KM401167	KM401267	KM401356
	Pseudomugil gertrudae	Unmack2013	n/a	n/a	n/a	KM401105	KM401208	n/a	n/a
	Pseudomugil novaeguineae	n/a	KM400909	KM400814	KM401007	KM401106	KM401209	n/a	KM401393
	Pseudomugil signifer	n/a	KM400910	KM400815	KM401008	KM401107	KM401210	n/a	KM401394
	Pseudomugil tenellus	JQ282014	n/a	n/a	n/a	n/a	KM401211	n/a	n/a
Telmatherinidae	*Kalyptatherina helodes*	KM400732	KM400867	KM400778	KM400964	KM401064	KM401165	KM401265	KM401355
	Marosatherina ladigesi	KM400733	KM400872	KM400783	KM400970	KM401070	KM401171	KM401271	KM401358
Outgroup taxa									
Beloniformes	*Ablennes hians*	AF231639	KM400819	KM400736	KM400914	KM401013	KM401112	KM401214	KM401312
	Platybelone argalus	AF243874	KM400905	KM400810	KM401003	KM401101	KM401204	KM401303	KM401389
	Oryzias latipes	AB480878	XM_004072598	n/a	XM_004069600.1	XM_004079893.1	EF032927	EF033005.1	n/a
Cyprinodonti-Formes	*Austrolebias arachan*	n/a	KM400844	KM400762	KM400941	KM401040	KM401141	KM401243	KM401337
	Poecilia latipinna	HQ677867	KM400908	KM400813	KM401006	KM401104	KM401207	KM401306	KM401392

(based on calibration 2, above). Prior setting: Exponential distribution, Mean = 4.34.

Calibration 4: *Atherinomorus stipes* new stem calibration for terminal branch. Hard minimum age: 6 Ma *A. stipes* (Nolf and Stringer, 1992). Diagnosis and phylogenetic placement: fossil otolith assigned to *A. stipes* by Nolf and Stringer (1992) based on otolith features. Stratigraphic horizon and locality: Cercado Formation, Dominican Republic. Absolute age estimate: 6 Ma, based on strontium isotope dating (McNeill et al., 2011); 95% soft upper bound of 23 Ma (based on calibration 2, above). Prior setting: Exponential distribution, Mean = 5.67.

Calibration 5: genus *Basilichthys* (new stem calibration). MRCA: *Basilichthys semotilus, Basilichthys australis.* Hard minimum age: 11 Ma *Basilichthys* sp. (Rubilar, 1994). Diagnosis and phylogenetic placement: maxilla with condyle on ventral process supports the placement of this fossil in *Basilichthys* (Dyer, 1997). Placed as a stem calibration due to lack of synapomorphies to define the species. Stratigraphic horizon and locality: Cura-Mallín Formation, Cerro La Mina and El Tallón, Chile. Absolute age estimate: 11 Ma, from K–Ar isotope dating (Suarez and Emparan, 1995); 95% soft upper bound 22 Ma (twice the age of the fossil). Prior setting: exponential distribution, Mean = 3.67.

Calibration 6: genus *Odontesthes* (new stem calibration). MRCA: *Odontesthes incisa*, *Odontesthes gracilis*. Hard minimum age: 20 Ma *Odontesthes* sp. (Bocchino, 1971). Diagnosis and phylogenetic placement: presence of opercular fenestration and no restriction in protractile premaxilla place this fossil within *Odontesthes* (Cione and Baez, 2007; Dyer, 1997). Stratigraphic horizon and locality: Ñirihuau Formation, Chubut, Argentina (Cione and Baez, 2007). Placed as a stem calibration due to lack of synapomorphies that define species. Absolute age estimate: 20 Ma, based on palynology and microfossils (Asensio et al., 2010); 95% soft upper bound 40 Ma (twice the age of the fossil). Prior setting: exponential distribution, Mean = 6.68.

Calibration 7: genus *Membras* (new stem calibration). MRCA: *Membras gilberti*, *Membras martinica*. Hard minimum age: 16 Ma *Membras* sp. (Nolf and Aguilera, 1998). Diagnosis and phylogenetic placement: otolith with posterior caudal end of sulcus curved dorsally supports the placement of the fossil in *Membras*. Placed in the stem due to lack of distinguishing synapomorphies for species. Stratigraphic horizon and locality: Cantaure Formation, Venezuela. Absolute age estimate: 16 Ma, lower bound of Early Miocene (Gradstein, 2012); 95% soft upper bound 32 Ma (twice the age of the fossil). Prior setting: exponential distribution, Mean = 5.34.

Four replicate searches were conducted with BEAST, each with 150 million generations. The log files were assessed for satisfactory mixing of the MCMC chains and effective sample size (ESS > 200) using Tracer v.1.5. The resulting outputs from the four independent runs were compiled with LogCombiner v1.8.0 and the maximum clade credibility tree was estimated with TreeAnnotator v1.8.0. The xml file used for this analysis is available at figshare.com as supplemental_data1 (See Appendix 2).

To gauge potentially misleading effects due to non-stationarity (e.g., Jermiin et al., 2004) we checked for base compositional bias using a chi-square test with the BaseFreq function implemented in PAUP* (Swofford, 2002). We also tested for substitution pattern disparity by calculating the average disparity index among taxa (Kumar and Gadagkar, 2001) for each of the 24 *a priori* data partitions using MEGA 5, following the approach described by Betancur-R et al. (2013b). Codon positions that significantly deviated from the homogeneity assumption were recoded for analysis in TreeFinder (Jobb, 2008) using AGY coding. TreeFinder is the only ML inference program that implements a GTR3 model to accommodate AGY-coded data. Since AGY coding results in loss of phylogenetic signal, only partitions with codon positions that deviated significantly from the homogeneity assumption were recoded in order to preserve as much signal in the data as possible. Taxa for which no more than two genes could be sequenced were eventually excluded from this recoding analysis, as loss of phylogenetic information caused their position in the tree to become unstable (see results).

2.3. Ancestral habitat reconstruction

Habitat information was downloaded for each of taxon in our data set from online compilations such as Catalogue of Fishes (Eschmeyer, 2013) and TreeBase (http://treebase.org), and also taken from metadata associated with collected specimens. We assigned each species to one of two possible states: marine (A) or freshwater (B). Euryhaline species known to tolerate a wide range of salinities and to undertake migrations into freshwater estuaries were coded as AB, since the model used for ancestral reconstruction allows species to occupy both types of habitats. Habitat data are provided in supplemental_data2 (available online at figshare.com, see Appendix 2) and are shown in Fig. 3. This information, along with the chronogram obtained with BEAST (available as supplemental_data3 at figshare.com, see Appendix 2), were uploaded to the Lagrange Configurator for analysis (http://www.reelab.net/lagrange/configurator), to estimate ancestral areas with Lagrange under a model with equal probability of transition between states (Ree and Smith, 2008).

3. Results

3.1. Sequence data, alignments, genetic divergence, and partitions

All DNA sequences obtained for this study have been deposited in GenBank and are listed in Table 2; these sequences passed the quality control step and were used for phylogenetic analysis. Not all loci could be amplified and sequenced for all taxa, and some sequences had to be discarded due to evidence of cross-contamination. As a consequence, each marker could be successfully sequenced for about 90% of the species (Tables 2 and 3), and only 60 taxa have a complete set of sequences for all markers. Species with the least amount of sequence data are *Atherina hepsetus* (with only two markers, *kbtbd4* and *sh3px3*), *Pseudomugil tenellus* (*cytb* and *myh6*), *Pseudomugil gertrudae* (*cytb*, *kiaa-l*, and *myh6*), and *Rheocles wrightae* (*cytb*, *myh6*, and *sh3px3*); all other taxa have sequences for four or more genes. The marker with the lowest rate of success was *gcs1*, missing in 23% of the taxa (Table 3). The average level of divergence among taxa for each marker ranged from 23% (*cytb*) to 5.6% (*sh3px3*), with most nuclear genes below or around 10% (Table 3). Therefore, the combined data set contains a diversity of rates of evolution among markers (and within markers due to codon positions) that needs to be accounted for in a partitioned analysis. The final concatenated alignment included 108 OTU, 6432 sites, and 12.3% invariable characters (available as supplemental_data4 at figshare.com, see Appendix 2). PartitionFinder analysis of the concatenated alignment resulted in eight partitions when searching under a greedy algorithm with the reduced set of models implemented in MrBayes (Table 4). This scheme was implemented in phylogenetic analyses using RAxML and MrBayes. PartitionFinder analysis based on all possible models resulted in a second partitioning scheme with 10 partitions (Table 4), which was used for analyses with Garli and BEAST. Results from the Chi Square test showed that the 3rd codon positions of *cytb*, *ficd*, *gcs1*, *kbtbd4*, *myh6*, and *slc10a3* differed significantly from base composition stationarity. Disparity index estimates were close to zero for all 1st and 2nd codon positions but showed average values between 1.2 and 4.5 for 3rd codon

Table 3
Summary of sequence data obtained, variation among taxa, and missing data.

Molecular marker	cytb	ficd	gcs1	kbtbd4	kiaa-l	myh6	sh3px3	slc10a3
Number of sequences	99	96	83	100	100	104	100	87
Alignment length (bp)	1104	645	1185	675	888	648	669	618
% Invariant sites	48	57.8	46.2	62.1	60.1	57.6	59	57.8
Inter-spp divergence[a]	23.6 (36.3)	11.1 (21.9)	12.3 (24.0)	8.1 (16.8)	6.5 (16.1)	9.7 (18.1)	5.6 (15.8)	9.3 (21.2)
Missing data[b]	8.3%	11.1%	23.1%	7.4%	7.4%	3.7%	7.4%	19.4%

[a] Divergence among species is measured as average and maximum (in parenthesis) percent sequence difference (percent *p*-distance).
[b] Percent of taxa (out of 108) missing sequence data for each marker.

Table 4
Partitioning scheme and best-fit models resolved by PartitionFinder (implemented in RAxML and BEAST analyses) and TreeFinder (for AGY-coded data). Partitions in bold were found to be non-stationary and were AGY-coded for analysis with TreeFinder under the GTR3 model.

PartitionFinder (MrBayes models for MrBayes and RAxML)			**PartitionFinder** (All models for Garli and BEAST)			**TreeFinder**		
Partition[a]	Data included	Model	Partition[a]	Data included	Model	Partition[a]	Data included	Model
1a	1st position ficd, gcs1, kbtbd4, kiaa-1, myh6, sh3px3, slc10a3	GTR + I + Γ	1b	1st position *gcs1*, *slc10a3*	K81uf + I + Γ	1c	1st position ficd, gcs1, kbtbd4, kiaa-1, myh6, sh3px3, slc10a3	GTR + Γ
2a	2nd position ficd, gcs1, kbtbd4, kiaa-1, myh6, sh3px3	GTR + I + Γ	2b	2nd position ficd, gcs1, kiaa-1, myh6, sh3px3	GTR + I + Γ	2c	2nd position ficd, gcs1, kbtbd4, kiaa-1, myh6, sh3px3	GTR + Γ
3a	**3rd position *gcs1*, *kbtbd4***, kiaa-1, sh3px3	GTR + Γ	3b	3rd position ficd, gcs1, kbtbd4, myh6	GTR + Γ	3c	**3rd position *gcs1*, *kbtbd4***	GTR3 + Γ
4a	**3rd position *ficd*, *myh6***	GTR + Γ	4b	1st position ficd, kbtbd4, kiaa-1, myh6, sh3px3	TIM + I + Γ	4c	**3rd position *ficd*, *myh6***	GTR3 + Γ
5a	2nd position *cytb*, *slc10a3*	GTR + I + Γ	5b	2nd position cytb, slc10a3	TVM + I + Γ	5c	2nd position cytb, *slc10a3*	GTR + Γ
6a	**3rd position *slc10a3***	GTR + Γ	6b	3rd position *slc10a3*	TVM + G	6c	**3rd position *slc10a3***	GTR3 + Γ
7a	1st position *cytb*	GTR + I + Γ	7b	3rd position kiaa-1, sh3px3	SYM + G	7c	1st position *cytb*	GTR + Γ
8a	**3rd position *cytb***	GTR + Γ	8b	2nd position *kbtbd4*	K80 + I	8c	**3rd position *cytb***	GTR3 + Γ
9a	–		9b	1st position *cytb*	TVMef + I + Γ	9c	3rd position *kiaa-1*, *sh3px3*	GTR + Γ
10a	–		10b	3rd position *cytb*	TrN + G	10c	–	

[a] 24 data blocks were defined *a priori*, by gene and codon position.

positions of all genes, except *sh3px3* and *kiaa-1*, which were close to 0.5. Therefore, the 3rd codon positions for all genes except *sh3px3* and *kiaa-1* were AGY-coded and analyzed under the GTR3 model with TreeFinder, with a ninth partition added to the first partitioning scheme to accommodate the stationary 3rd codon positions (Table 4). All stationary partitions were analyzed in TreeFinder under the GTR + Gamma model.

3.2. Phylogenetic relationships

RAxML (Fig. 2), Garli, and MrBayes searches all resulted in congruent topologies, but BEAST produced a slightly different result (Fig. 3). Analyses using BEAST produced ESS values >200 for the combined runs, although some ESS values were low for partitions that contained only a single codon position. Trees sampled for the first 20 million generations of each run were discarded as *burn-in*. Newick files for trees obtained with BEAST and RAxML are available as supplemental_data3 and supplemental_data5, respectively, at figshare.com (see Appendix 2). Analysis with TreeFinder based on the AGY-coded data for non-stationary 3rd codon partitions (Table 4), resulted in almost the same topology obtained by RAxML when two rogue taxa (*Atherina hepsetus* and *Pseudomugil tenellus*) were excluded. Only two gene partitions were available for these two taxa, therefore loss of information due to AGY coding was likely the cause of erroneous placement with unrelated taxa in different families across replicate runs. The only difference between the topology obtained with RAxML and TreeFinder (when excluding the two rogue taxa) involves branching order within the *Odontesthes argentinensis* clade and the relative positions of *Menidia colei* and *Menidia menidia*. The newick file with TreeFinder results is available as supplemental_data6 at figshare.com (see Appendix).

All analyses resolve with high support the subdivision of Atheriniformes in two suborders, with *Notocheirus hubbsi* nested within the family Atherinopsidae. Within this family, minor differences in branching pattern for the ML and Bayesian results were observed for *Poblana*, some species of *Chirostoma*, and for the position of *Menidia menidia*, but all analyses support the monophyly of the tribe Menidiini (*Chirostoma*, *Labidesthes*, *Menidia*, and *Poblana*) with *Labidesthes sicculus* as the sister group to the rest of the taxa in this clade (Figs. 2A and 3). In contrast, the tribe Membradini (*Atherinella*, *Melanorhinus*, *Membras*) was not resolved as a monophyletic group since *Melanorhinus microps* is more closely related to Menidiinae than to species of *Atherinella*, and *Atherinella brasiliensis* is the sister group to all other members of the subfamily Menidiinae (Figs. 2A and 3). At the generic level, there is no support for the monophyly of *Atherinella*, *Chirostoma, Menidia*, or *Poblana* as currently defined, suggesting necessary revisions to the taxonomy. *Notocheirus hubbsi* was unambiguously resolved as the sister group of Menidiinae.

Less congruence between ML and Bayesian results was observed for relationships among families in the suborder Atherinoidei (Figs. 2B and 3). For example, Isonidae was resolved either as the sister group to Atherinidae (RAxML) or as the sister group to a larger clade that contains Atherinidae, Bedotiidae, Melanotaeniidae, Pseudomugilidae, and Telmatherinidae (BEAST). All analyses failed to support the monophyly of Melanotaeniidae because *Cairnsichthys* is never in the same clade as the other taxa

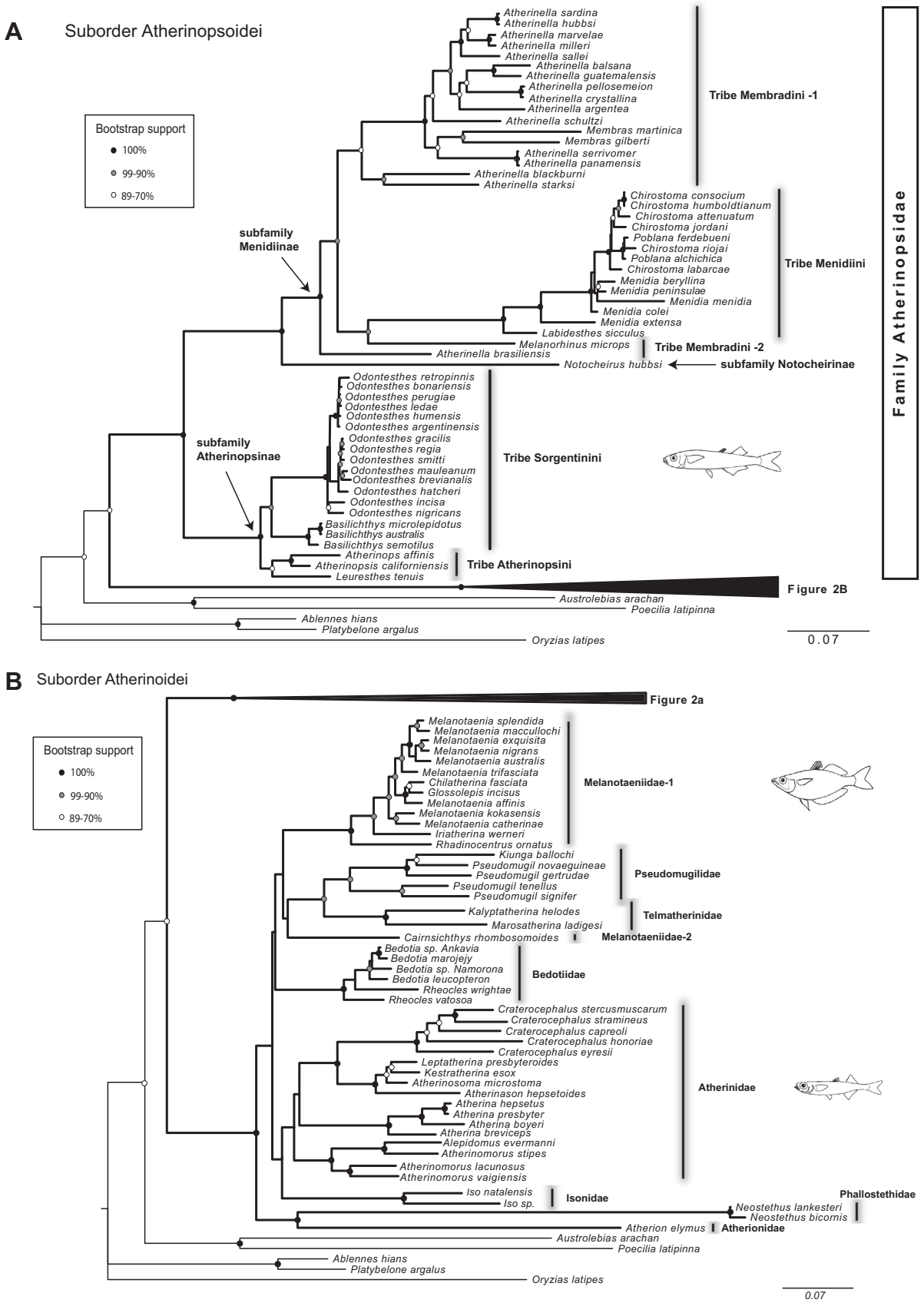

Fig. 2. Maximum-likelihood phylogenetic hypotheses for the Atheriniformes obtained with RAxML. Bootstrap support values are indicated on nodes as black (100% bootstrap support), gray (99–90%) and white circles (89–70%). A: suborder Atherinopsoidei; B: suborder Atherinoidei.

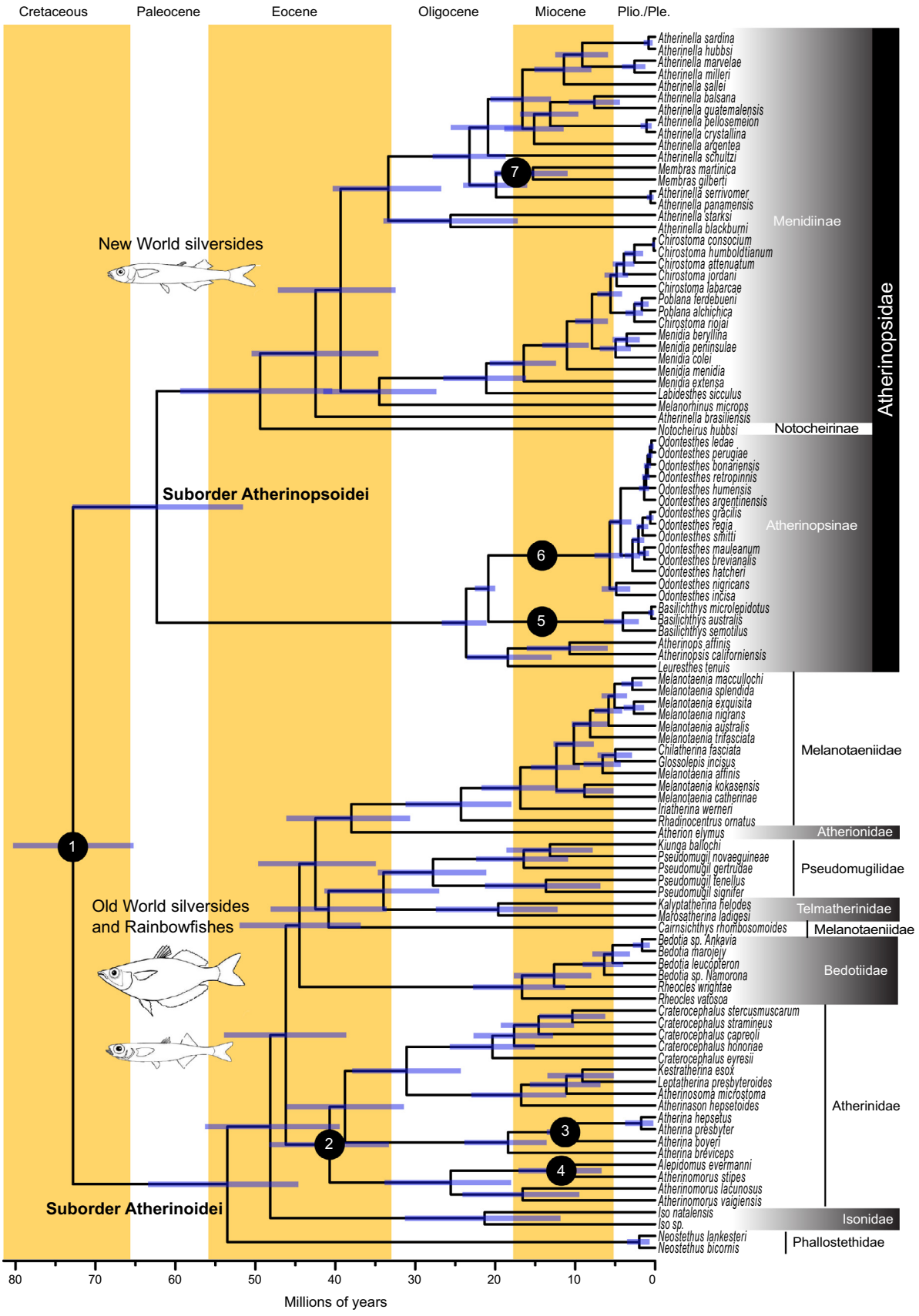

Fig. 3. Time-calibrated phylogeny obtained with BEAST. Numbers inside black circles indicate the placement for the 7 calibrations used. Bars represent the 95% highest posterior credibility intervals of divergence times.

included in this family. The position of Bedotiidae in relation to Melanotaeniidae, Pseudomugilidae and Telmatherinidae is not resolved with confidence, but there is strong support for a sister group relationship between Pseudomugilidae and Telmatherinidae (Figs. 2B and 3). RAxML placed Phallostetids and *Atherion* in a well supported clade that is sister to all other taxa in the suborder Atherinoidei (Fig. 2A), but BEAST results placed *Atherion* as the sister taxon to Melanotaeniidae, to the exclusion of *Cairnsichthys* that is now placed as the sister group of Telmatherinidae plus Pseudomugilidae (Fig. 3). Our results support the monophyly of the family Atherinidae, its subfamilies Craterocephalinae, Atherinomorinae and two genera for which we had more than one species in our data set: *Craterocephalus* and *Atherina*.

In spite of the discrepancies mentioned above, the data set provided significant phylogenetic signal to resolve relationships within Atheriniformes. A test of alternative hypotheses (Shimodaira and Hasegawa, 2001) rejected the topology proposed by Dyer and Chernoff (1996), summarized in Fig. 1A ($p < 0.001$). Atherinopsidae and its subfamilies were well supported, with 100% bootstrap support in RAxML, and 0.9–1.0 posterior probability in BEAST. Posterior probability density values on the consensus BEAST tree were higher than 0.9, both at the ordinal and family levels. However, our dataset provided weak resolution for some relationships among families in Atherinoidei. While placement of Phallostethids as sister to all other taxa is highly supported (posterior probability of 1), the relationships among other families are not, ranging from 0.24 for the relationship between Atherinidae and Bedotiidae, Telmatherinidae, Pseudomugilidae, *Atherion* and Melanotaeniidae, to 0.55 for the relationship between Isonidae and all other families excluding Phallostethids. The phylogenetic placement of *Atherion* is still unresolved, as our maximum likelihood analysis placed it sister to the Phallostethids with high support, but BEAST placed it as sister to Melanotaeniidae, although with low posterior probability.

3.3. Time-calibrated phylogeny

Fig. 3 shows the time-calibrated phylogeny obtained with BEAST, indicating the position of six fossil calibration points and one secondary calibration for the root. Divergence times and their estimated 95% highest posterior density (HPD) intervals place the origin of this order in the Late Cretaceous (72.8 Ma). Subsequent divergence between Old World and New World taxa started in the Paleogene and all currently recognized families originated during the Eocene and Oligocene (50–23 Ma).

3.4. Ancestral habitat reconstruction

Reconstruction of habitat occupancy indicates, with a 37% relative probability, that the common ancestors to the New and Old World lineages, Atherinopsoidei and Atherinoidei, were both marine. However, this analysis also suggests (with 30% probability) that the Old World ancestor could have been euryhaline, and a 13% probability of a freshwater ancestor. Within Atherinopsidae, the ancestors of subfamilies Atherinopsidae and Menidiinae were reconstructed as marine with a high probability (76%). Highest probability values for reconstructed ancestral habitats are indicated for all nodes in Fig. 4. Complete results of the Lagrange analysis can be found in supplemental_data7 at figshare.com (see Appendix 2). We also performed habitat reconstruction on a calibrated time-tree constrained to the RAxML topology, to account for different scenarios due to our conflicting topologies in Atherinoidei. The atheriniform ancestor is reconstructed as marine with a higher probability (60%) under this constrained topology (complete results can be found in supplemental_data8 at figshare.com, Appendix 2).

4. Discussion

This study provides a comprehensive time-tree for the order Atheriniformes. Our dataset includes 103 atheriniform species, almost 30% of the 352 valid species in the order, a significant increase from previous molecular phylogenies where the species coverage varied from 1% to 14% (Bloom et al., 2012; Setiamarga et al., 2008; Sparks and Smith, 2004). We included 2/3 of all genera, with dense sampling in the most genera-rich families (92% for Atherinopsidae, 67% for Atherinidae, 86% for Melanotaeniidae). Unfortunately, *Dentatherina merceri* (monotypic family Dentatherinidae) was not available for this study nor included in any of the published molecular phylogenies, hence its relationships remain unresolved. The amount of DNA sequence data analyzed herein also is larger than previous efforts, with a total length of 6432 sites for eight gene fragments (one mitochondrial and seven nuclear loci), compiled into a data matrix that is 89% complete (Tables 2 and 3). The resulting molecular phylogeny was calibrated on the basis of six carefully documented fossil taxa, placed on the phylogeny with high confidence. Phylogenetic resolution afforded by our data set was significant, with high measures of support for most internal branches, and substantial convergence among different types of analyses (ML and Bayesian, but see below). Species trees methods that account for coalescent variance to accommodate potential biases due to anomalous gene tree distributions (e.g., Huang et al., 2010) were not tested. In order to obtain a dataset with complete gene representation for all families suitable for *BEAST (Drummond et al., 2012), our matrix would have to be reduced to five markers (out of eight) and 36 ingroup species (out of 103). We preferred to emphasize taxon sampling and maximal use of our phylogenetic markers, a strategy that provides robust results for inference of deep phylogenetic questions with concatenation approaches (Lambert et al., 2014). Potential biases originating from non-stationarity of base composition in some data blocks (most 3rd codon positions) were shown to have no effect on phylogenetic results. The time-calibrated phylogeny presented in Fig. 3 provides an explicit framework for understanding the evolution of this important group of fishes.

4.1. Taxonomic implications

The early split between New World and Old World silversides suggested by several authors (Fig. 1) was resolved with confidence in this study, supporting the subdivision of Atheriniformes in two suborders (Atherinopsoidei and Atherinoidei). Phylogenetic resolution within Atherinopsoidei afforded by our data was more robust than within Atherinoidei, as indicated by lower bootstrap and posterior probability values and by discordances in topology between ML and Bayesian results for Atherinoidei (Figs. 2B and 3). Taxonomic sampling also was more robust within Atherinopsoidei (49% or 54 out of 110 species) than Atherinoidei (20% or 49 out of 242 species). As a consequence, taxonomic recommendations for Atherinoidei seem somewhat premature on the basis of our data.

Our results for Atherinopsoidei are consistent with a previous study (Bloom et al., 2012) that resolved the position of *Notocheirus hubbsi* among New World taxa. In agreement with these authors, we support the designation of a monotypic subfamily Notocheirinae within Atherinopsidae, placed as the sister-group of the subfamily Menidiinae. A third subfamily (Atherinopsinae) is supported with confidence by our data.

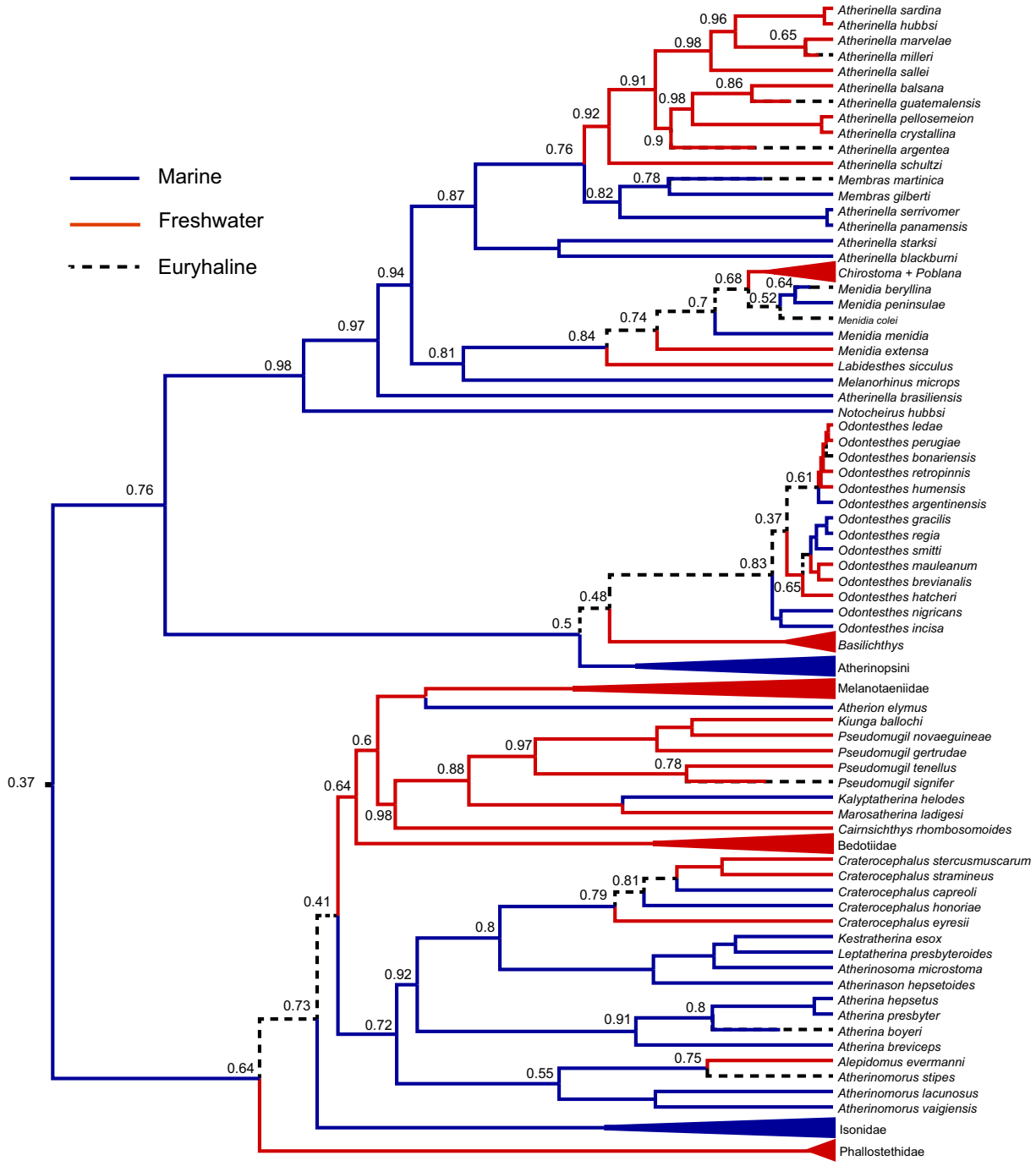

Fig. 4. Ancestral habitat reconstruction for the Atheriniformes based on the chronogram obtained with BEAST (Fig. 3). Marine state indicated by blue lines, freshwater by red, and euryhaline by black dotted lines. Probability values on the nodes indicate the probability of the reconstructed ancestral state shown. Euryhaline taxa that do not have a euryhaline ancestral are shown with their most probable ancestral state colored at the subtending node. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Within Menidiinae, the monophyly of the tribe Membradini proposed by Chernoff (1986) and Dyer and Chernoff (1996) is not corroborated by our results (Figs. 2A and 3). *Melanorhinus microps*, placed by these authors within Membradini, has closer affinities with tribe Menidiniini (labeled Membradini-2 in Fig. 2A), a result also supported by analyses of *rag1* and *cytb* data (Bloom et al., 2012). Two other species of *Melanorhinus* (*M. boekei* and *M. cyanellus)* were not available for either of the studies, but a redefinition of Menidiini that contains the genus *Melanorhinus* seems necessary. Within Menidiini, our analyses support sinking *Chirostoma* Swainson 1839 and *Poblana* de Buen 1945 as junior synonyms of *Menidia* Bonaparte 1836, in agreement with Miller et al. (2005). Another taxon previously assigned to Membradini (*Atherinella brasiliensis*) is placed with confidence as a sister group to all other taxa in the Menidiinae, a result also obtained by analysis of mtDNA (*nd2* and *cytb*) and two nuclear genes, *tmo4c4* and *rag1* (Bloom et al., 2013), suggesting that a new tribe may need to be defined for this taxon and putative close relatives in the future. This result also implies that the genus *Atherinella* is in need of revision. In fact, since the two species of *Membras* (*M. gilberti* and *M. martinica*) are

deeply nested among all species of *Atherinella* included in this study (except *A. brasiliensis*) it is necessary to reassign all species in the clade labeled Membradini-1 (Fig. 2A), which includes the type species for the genera *Atherinella* (*A. panamensis* Steindachner 1875) and *Membras* (type species for *Membras* is *M. martinica* Bonaparte 1836), to the genus *Membras*. Therefore, *Atherinella* becomes a junior synonym of *Membras*. *Atherinella brasiliensis* (Quoy and Gaimard, 1825) should be reassigned to the genus *Xenomelaniris* (Shultz, 1948), formerly a subgenus of *Atherinella*, changing its valid name to *Xenomelaniris brasiliensis* (Quoy and Gaimard, 1825). Other species that may be included in *Xenomelaniris* but were not examined in this study include *Atherinella robbersi* from Lake Totumo, Colombia and *A. venezuelae* from Trinidad & Tobago and Venezuela, that also were placed in the subgenus *Xenomelaniris* by Chernoff (1986). Until a complete taxonomic revision of these two latter species is completed we do not assign these taxa to any tribe and list them, together with *X. brasiliensis*, as *insertae sedis* within Menidinnae. Table 5 lists these proposed changes in a sequential classification.

Within Atherinoidei, some of our results are congruent with previous hypotheses, such as the non-monophyly of Melanotaeniidae (Aarn and Ivantsoff, 1997; Bloom et al., 2012), supporting the notion that *Cairnsichthys* should be recognized as an independent lineage. When initially described, *Cairnsichthys* was placed as a sister group to Pseudomugilidae based its morphological specializations (Allen, 1980); it was later resolved as sister to Pseudomugilidae + Telmatherinidae (Bloom et al., 2012), or to the rest of Melanotaeniidae (Unmack et al., 2013). The morphological distinctiveness of *Cairnsichthys* has been attributed to its restricted distribution in a few drainages in the wet Tropics of Queensland, to competition with sympatric *Melanotaenia splendida*, or to intense predation pressure (Unmack et al., 2013). We tentatively list *Cairnsichthys* as *insertae sedis* within Atherinoidei (Table 5). Within Melanotaeniidae, our phylogenetic hypothesis are consistent with previous results proposing the non-monophyly of *Chilatherina, Glossolepis,* and *Melanotaenia* (Unmack et al., 2013). Geographic groups were proposed for species of *Melanotaenia* by these authors, distinguishing Western New Guinea (*M. catherinae* and *M. kokasensis*), Northern New Guinea (*C. fasciata, G. incisus* and *M. affinis*), and Southern New Guinea-Australia (*M. australis, M. exquisita, M. maccullochi, M. nigrans, M. splendida, M. trifasciata*). Both our RAxML and BEAST results support these groupings, though there is minor conflict between the topologies of the Southern New Guinea-Australian clade (Figs. 2B and 3).

Other phylogenetic results within Atherinoidei receive consistent support and may be informative for taxonomy. For example, an earlier suggestion to sink Telmatherinidae into Pseudomuglidae (Sparks and Smith, 2004), also consistent with analysis of mtDNA data alone (Stelbrink et al., 2014), is not supported by our results. Both families are resolved as monophyletic groups with high confidence and placed as sister taxa in all our analyses (Figs. 2B and 3). The revised classification for rainbowfishes proposed by these authors (Sparks and Smith, 2004: their Table 3) is based on fewer taxa for these two families or on a single molecular marker (Stelbrink et al., 2014). On the other hand, their sampling within the family Bedotidae included 18 OTUs for *Bedotia* and six for *Rheocles* resulting in the non-monophyly of the latter. This hypothesis is consistent with our results, supporting their recommendation for genus *Rheocles* to be retained for *R. wrightae* (plus *R. alaotrensis* and *R. lateralis*) and to erect a new genus for *R. vatosoa* (and *R. derhami*). The suborder Melanotenoidei (containing Pseudomugilidae, Melanotaeniidae, Bedotidae) is not supported by any of our analyses.

Table 5
New sequential classification of families of Atheriniformes and subfamilies, tribes, and genera of Atherinopsidae based on phylogenetic relationships proposed herein (Fig. 2).

Order Atheriniformes Rosen 1966
Suborder Atherinopsoidei
Family **Atherinopsidae** Fitzinger 1873
Subfamily **Atherinopsinae** Fitzinger 1873
Tribe **Atherinopsini** Fitzinger 1873
Atherinops Steindachner 1876
Atherinopsis Girard 1854
Colpichthys Hubbs 1918 (not examined)
Leuresthes Jordan & Gilbert 1880
Tribe **Sorgentinini** Pianta de Risso & Risso 1953
Basilichthys Girard 1855
Odontesthes Evermann & Kendall 1906
Subfamily **Menidiinae** Schultz 1948
Insertae Sedis within Menidiinae: *Xenomelaniris brasiliensis* (Quoy & Gaimard 1825), "*Atherinella*" *venezuelae* (not examined) and "*Atherinella*" *robbersi* (not examined)
Tribe **Menidiini** Schultz 1948
Labidesthes Cope 1870
Melanorhinus Metzelaar 1919
Menidia Bonaparte 1836 [includes *Chirostoma* Swainson 1839 and *Poblana* de Buen 1945]
Tribe **Membradini** Chernoff 1986
Membras Bonaparte 1836 [includes *Atherinella* Steindachner 1875]
Subfamily **Notocheirinae** Schultz 1950
Notocheirus Clark
Suborder Atherinoidei
Insertae sedis within Atherinoidei: *Cairnsichthys* Allen 1980
Family **Atherinidae** Risso 1827
Family **Atherionidae** Schultz 1948
Family **Bedotiidae** Jordan & Hubbs 1919
Family **Isonidae** Rosen 1964
Family **Melanotaeniidae** Gill 1894
Family **Phallostethidae** Regan 1916
Family **Pseudomugilidae** Kner 1867
Family **Telmatherinidae** Munro 1958

4.2. Timing of diversification

The few published time-calibrated atheriniform phylogenies available have been inferred for smaller subset of taxa and were based on single calibration points or on estimated rates of molecular divergence. For example, the origin of five European *Atherina* species (*A. boyeri*, *A. breviceps*, *A. hepsetus*, and *A. presbyter*) was placed at 19 Ma (Pujolar et al., 2012) assuming that a paleogeographic event (closure of the Gibraltar strait dated 5.6 Ma) caused vicariance between *A. hepsetus* and *A. presbyter*. This estimate, however, is remarkably close to our estimate for this node (MRCA of *A. hepsetus* and *A. breviceps*) at ~19 Ma and consistent with the 10 Ma †*Atherina atropatiensis* used for calibration point 3 (Carnevale et al., 2011). Bloom et al. (2013) calibrated a molecular phylogeny for New World silversides (Atherinopsidae) using three fossil constraints and obtained a date of 37 Ma for the origin of this family and 27 Ma for the origin of Menidiinae. These dates are significantly younger than our estimates of 62 Ma and 42 Ma for Atherinopsidae and Menidiinae, respectively (Fig. 3). Their younger age estimates are a likely consequence of misinformative fossil priors, most critically the hard minimum bound of 5.3 Ma for the MRCA of *Basilichthys* and *Odontesthes* (Bloom et al., 2013: 2042), given that stratigraphic studies place the age of *Basilichthys* and *Odontesthes* fossils at 11 and 20 Ma, respectively (Suarez and Emparan, 1995), as used for our calibration points 5 and 6 (see methods). Bloom et al. (2013) also have reduced taxonomic representation within Atherinoidei (only *Atherinomorus* was used as an outgroup) precluding definition of fossil constraints within this suborder. Another study focusing only on Melanotaeniidae (Unmack et al., 2013) assumed a "standard" pairwise divergence rate of 1% for the cytochrome b gene to date the origin of this family at ~80 Ma (95% HPD of 63.5–99 Ma). This

age is significantly older than our estimate for the origin of Melanotaeniidae (24 Ma, Fig. 3), and still older than our estimated date for the origin of Atheriniformes (72.8 Ma). Another study based on mtDNA alone (Stelbrink et al., 2014) used the same rate of evolution for cytb and three alternative calibration approaches and inferred the origin of Melanotaeniidae at 17–55 Ma, encompassing our estimated value of 23 Ma. Molecular rates are highly variable among taxa and therefore not reliable as "standard yardsticks" to calibrate phylogenies, diminishing confidence in these results. It also may be argued that the age prior for the root of Atheriniformes used in our analysis (calibration 1: 70.5 Ma, 95% soft upper bound 77.5 Ma) imposed a strong constraint on inferred maximum ages. This root prior, however, was based on large scale-analysis of 202 taxa representing all major bony fish lineages with 60 fossil calibration points (Betancur-R et al., 2013a). The most relevant fossils among the 60 used in that study were two 49 Ma heroine and geophagine cichlids (López-Fernández et al., 2013), phylogenetically close to atheriniforms within Ovalentaria. Therefore, the weight of evidence used to support our choice or root calibration prior and consistency with other fossils used in our study increase confidence in our results. The divergence date estimated by Unmack et al. (2013) for Melanoteniidae is closer to the age of Ovalentaria estimated by Betancur-R et al. (2013a) and others (~100 Ma). This issue is critical to assess competing biogeographic hypotheses, for example to explain the current distribution of freshwater melanoteniids and bedotiids (see discussion on ancestral habitat reconstruction, below).

4.3. Vicariance, oceanic dispersal, and freshwater invasions in Atheriniformes

Though vicariance has long been the leading explanation for widely distributed taxa (Parenti, 2008), improved methodologies in sequencing, molecular phylogenetics, and time-calibrated phylogenies have found support for dispersal as a more likely explanation for the distribution of many groups (Crisp et al., 2011; de Queiroz, 2005; Sanmartin, 2008). The dates of divergence among families obtained here post-date significantly Gondwanan continental break-up events invoked by most vicariance hypotheses (e.g., Sparks and Smith, 2004; Unmack et al., 2013). Therefore, the current distribution of Atheriniformes is more likely the result of oceanic dispersal. This hypothesis is consistent by the wide range of salinity tolerance displayed by atheriniform fishes, making marine dispersal physiologically plausible, and by evidence that euryhalinity has evolved multiple times in some atheriniform groups (Bloom et al., 2013). We find additional support for a marine-dispersal hypothesis in the results of ancestral habitat reconstruction, which suggests that the atherinopsoid and atherinoid ancestors were marine or euryhaline (see Fig. 4, and supplemental_data7 and 8 for more detail), implying that the divergence between Atherinopsoidei and Atherinoidei is the result of marine dispersal.

The marine–freshwater boundary poses a significant physiological challenge to many organisms (Lee and Bell, 1999; Bloom and Lovejoy, 2012), but silverside fishes seem to cross it with relative ease. Our fossil-calibrated phylogeny (Fig. 3) in combination with the ancestral habitat reconstruction (Fig. 4, supplemental_-data 7 and 8) points to several instances of marine dispersal and subsequent freshwater colonization by silversides. For example, the distribution of the atherinid species *Alepidomus evermanni* and *Atherinomorus stipes* in the Caribbean can only be explained by marine dispersal, followed by freshwater invasion by *A. evermanni*, given that their closest relatives inhabit the Indian and western Pacific Oceans. Similarly, the divergence between Melanotaeniidae and Bedotiidae was hypothesized to be the result of vicariance following the break-up of Gondwana, 140–80 Ma (Sparks and Smith, 2004; Unmack et al., 2013). We do not find these families as sister taxa in either of our analyses, though our ancestral habitat reconstruction suggests a freshwater ancestor for each of the Bedotiidae, Pseudomugilidae, Telmatherinidae, and Melanotaeniidae families. This is possibly due to the exclusively freshwater habitat of modern taxa found in Bedotiidae and Melanotaeniidae and lack of any fossil evidence that would provide data on historical ranges that could imply marine habitat use. The topology obtained in BEAST does suggest a possible relationship between *Atherion elymus*, a marine species, and the freshwater Melanotaeniids (excluding *Cairnsichthys*), but this relationship is poorly supported in our analyses. At 44 Ma (Fig. 3), the ancestor of all of these families is too young to explain the current distribution of these taxa by vicariance. Instead, we hypothesize that extinct marine ancestors must have dispersed and colonized freshwater. In the suborder Atherinopsoidei, where taxon sampling is more complete, repeated invasions of freshwater by marine or euryhaline ancestors are relatively common, occurring in *Atherinella*, *Chirostoma* and *Poblana*, *Basilichthys* and *Odontesthes*. Frequent marine dispersal followed by freshwater colonization make Atheriniformes an interesting system in which to study the processes of speciation and diversification along the marine–freshwater barrier (Bloom et al., 2013).

In addition to marine dispersal, two vicariant events have been hypothesized to be responsible for the current distribution of atherinopsoid silversides: the rise of the Isthmus of Panama, and equatorial warming during the middle Miocene (White, 1986). Menidiinae is distributed in Central America in both the Atlantic and Pacific Oceans, with some species extending northwards along eastern North America. Our study and others (Bloom et al., 2013) do not find support for the hypothesis that the distribution of Menidiinae is a result of vicariance due to the rise of the modern Isthmian link, which rose above sea level 3.5 Ma (Coates et al., 1992). However, an earlier Isthmian link that formed during the Eocene and may have persisted into the Miocene has been suggested by geological data (Montes et al., 2012). Our confidence intervals on the divergence between Pacific-distributed "*Atherinella*" *blackburni* and "*Atherinella*" *starksi* from their Atlantic relatives extends into the upper Eocene (~33 Ma, Fig. 3), so we cannot rule out vicariance caused by an older Isthmian link. Similarly, the anti-tropical distribution of Atherinopsinae was proposed to be the result of unfavorable climatic warming in the Middle Miocene (White, 1986). Our analysis dates the origin of Atherinopsinae to the Oligocene, making the vicariance scenario unlikely. Ultimately, we do not have conclusive evidence for a vicariance hypothesis in New World silversides, but do find that oceanic dispersal was a primary driver in shaping the modern distribution of Atheriniformes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.03.001.

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