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Short Communication

It's a family matter: Molecular phylogenetics of Atheriniformes and the polyphyly of the surf silversides (Family: Notocheiridae)

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ABSTRACT

Phylogenetic relationships among families of Atheriniformes have long been problematic. The affinities of one of the most enigmatic lineages, surf silversides (Notocheiridae), have proven particularly elusive due to this taxon's unique morphology and rarity in museum collections. In this study, we use mitochondrial and nuclear sequence data to generate a phylogeny for seven of the eight families of Atheriniformes. Our results reveal that four families within Atheriniformes (Atherinopsidae, Notocheiridae, Atherinidae, Melanotaeniidae) are not monophyletic. Most notably, Notocheiridae is polyphyletic, with *Notocheirus hubbsi* nested within New World silversides (Atherinopsidae), while members of *Iso* are sister to all other Old World Atheriniforms. These data suggest that the unique morphology of *Notocheirus* and *Iso* is a result of adaptive convergent evolution to the high-energy surf habitat where these species live.

1. Introduction

Atheriniformes are a clade of predominantly surface dwelling fishes that occur throughout tropical and temperate regions. The eight families and >300 species that comprise Atheriniformes are widespread, abundant and ecologically important forage fishes in near-shore marine, estuarine, and freshwater habitats. Atheriniform relationships have been considered using molecular (Setiamarga et al., 2008) data, morphological data (Saeed et al., 1994; Dyer and Chernoff, 1996), and combined molecular/morphological approaches (Sparks and Smith, 2004). In addition, several studies have investigated molecular relationships within families or smaller species groups (Beheregaray et al., 2002; Bloom et al., 2009; McGuigan et al., 2000; Roy et al., 2007; Sparks and Smith, 2004; Unmack and Dowling, 2010). Currently, the monophyly and relationships among families within Atheriniformes remain uncertain. Indeed, in his seminal treatise on fish systematics, Nelson (2006) concluded that determining inter-familial relationships is a "major need" of Atheriniform systematics.

One of the more intriguing questions regarding Atheriniform family relationships is the placement, composition and monophyly of the family Notocheiridae, commonly known as the surf silversides. As defined by Dyer and Chernoff (1996), Notocheiridae is comprised of two marine genera: *Notocheirus* (one species) and *Iso* (five species). While many Atheriniforms species have an elongate missile shaped body that is well adapted to the calm water surface water habitats inhabited by most members of this group, notocheirids have a deep and highly laterally compressed body shape, along with pectoral fins positioned high on the body (Saeed et al., 2006), and these features are thought to represent adaptations to the high-energy surf habitats inhabited by members of this clade. Notocheirids also have a highly disjunct geographic distribution; the monotypic *Notocheirus hubbsi* is found along the Pacific and Atlantic coasts of Southern South America, while *Iso* consists of five species found in the Indo-Pacific. This distribution is unusual among Athernifoms, where most families are restricted to either the Old World (Melanotaeniidae, Phallostethidae, Pseudomugilidae, Bedotiidae, Atherionidae, Telmatherinidae) or the New World (Atherinopsidae).

To date, the phylogenetic position and composition of the notocheirid surf silversides has only been studied using morphology. Saeed et al. (1994) recognized both *Iso* and *Notocheirus* at the family level (Isonidae and Notocheiridae) and proposed a sister relationship between New World silversides (Atherinopsidae) and *Iso* + *Notocheirus*. This clade was considered the sister group to all other Atheriniformes, Beloniformes and Cyprinodontiformes, rendering Atheriniformes a paraphyletic assemblage (Fig. 1a).

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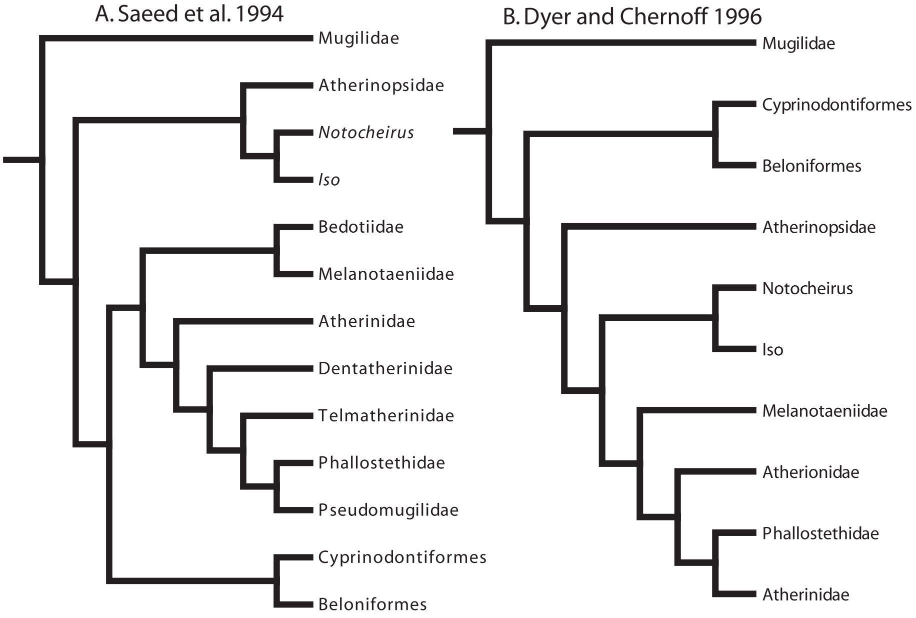

Fig. 1. Previous phylogenetic hypotheses for Atheriniformes by (A) Saeed et al. (1994; these authors included *Atherion* in Atherinidae) and (B) Dyer and Chernoff (1996).

However, Saeed et al.'s (1994) hypothesized grouping of *Iso* + *Notocheirus* with Atherinopsidae was based on a single character, the spine-like dorsolateral process of the pelvic girdle. Dyer and Chernoff (1996) studied Atheriniform relationships using osteological characters and found Atherinopsidae to be sister to all other Atheriniformes including notocheirids. Notocheirids were proposed as the basal lineage with the latter clade (Fig. 1b). Importantly, both Saeed et al. (1994) and Dyer and Chernoff (1996) supported a monophyletic Notocheiridae, differing only on the position of this lineage within Atherinomorpha (Atheriniformes, Cyprinodontiformes, Beloniformes). However, the disjunct distribution of notocheirids, combined with their specialized adaptive morphology, raises the possibility that the clade may not be monophyletic. Instead, could *Notocheirus* and *Iso* represent lineages that have independently adapted to the surf environment in different biogeographic regions?

Collections of notocheirids are exceedingly rare (Dyer, 2006) and tissues have not previously been available for molecular analysis. This has prevented a much-needed molecular investigation of Atheriniform relationships to test the placement of *Notocheirus* and *Iso*. Here we present a new molecular phylogeny of Atheriniformes that is based on >2.5 kb of mitochondrial and nuclear data and includes both *Notocheirus* and *Iso*. Our objectives are to: (1) provide a molecular phylogenetic hypothesis for Atheriniformes, (2) test the monophyly of Notocheiridae and other Atheriniform families, and (3) test the previous morphology-based hypotheses for the phylogenetic placement of notocheirids.

2. Materials and methods

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

Our data set consists of 47 ingroup species from 26 genera and seven of the eight families in Atheriniformes (missing only the monotypic Atherionidae; Table 1). Preliminary data suggested that *Notocheirus* grouped with Atherinopsidae, thus we expanded our taxon sampling of Atherinopsidae to include most genera (11 out of 13 genera). We included 10 outgroup taxa, representing Cyprinodontiformes, Beloniformes, and Mugiliformes which are likely sister groups to Atheriniformes (Stiassny, 1990; Parenti, 1993; Setiamarga et al., 2008). Trees were rooted with *Ambassis* (Ambassidae).

Total genomic DNA was isolated using DNeasy® blood and tissue kit (Qiagen, Valencia, CA). An approximately 1500 bp fragment of the third exon from the nuclear gene RAG1 (recombination activating gene one) was PCR amplified. For Atherinopsidae, samples were amplified using primers RAG1F1 5'-ATCCTGCTGTGTGTTTGGCYAT-3' and RAG1_1441R 5'-GACTGACGKGCATTCATCTTCC-3'. Remaining samples were amplified using the primers listed in Unmack and Dowling (2010). The entire cyt*b* (cytochrome *b*) gene was amplified in Atherinopsidae using primers in Lewallen et al. (2011) while the other samples were amplified with Glu31-RF.Thr.48 (Unmack and Dowling, 2010). PCR reactions for both genes consisted of 5 μl 10× PCR buffer, 4 μl $MgCl_2$, 2 μl dNTPs (10 mM), 2 μl each primer (10 mM), 1 μl Taq polymerase, 1–5 μl genomic DNA, and the remaining volume consisting of H_2O for a total of 50 μl. Thermocycling conditions were 95° for 2 min, 35–40 cycles of 95° for 1 min, 50–55 for 1 min, 72° for 90 s, and 72° for 5 min. Amplified PCR products were purified using a Qiagen PCR purification kit. Sequences were edited and aligned using Sequencher 4.6 (Genecodes) and Geneious v5.4 (Drummond et al., 2010).

2.2. Phylogenetic analysis

We analyzed the cyt*b* and RAG1 genes independently using maximum parsimony (MP) and maximum likelihood (ML). We also concatenated the two genes into a single matrix and generated phylogenies using MP, ML, and Bayesian inference (BI). MP analyses were conducted in PAUP∗ 4.0b10 (Swofford, 2003) and consisted of un-weighted heuristic searches using 10,000 random sequence addition replicates and TBR branch swapping, with support for maximum parsimony trees generated from 1000 bootstrap replicates. ML tree searches were conducted using GARLI v0.95 (Zwickl, 2006). We implemented 10,000 heuristic replicates using the GTR + G + I model of sequence evolution and allowed base frequencies to be estimated from the data. ML bootstrap support values were obtained from 100 bootstrap replicates. For BI, we used a partitioned mix-model approach where the partitions were defined by codon positions for each gene. Best-fit models of sequence

Table 1
Taxon sampling and Genbank accession numbers for specimens used in this study.

Family	Taxon	Genbank accession number	
		RAG1	CytB
Atherinidae	*Craterocephalus eyresii*	JQ282042	GU932886
	Craterocephalus mugiloides	JQ282043	GU932767
	Craterocephalus stramineus	JQ282044	GU932791
	Hypoatherina harringtonensis	JQ282045	GU932756
	Hypoatherina temminkii	JQ282046	GU932757
	Kestratherina esox	JQ282050	GU932762
Atherinopsidae	*Atherinella argentea*	JQ282062	JQ282017
	Atherinella guatamalanensis	JQ282064	JQ282019
	Atherinella hubbs	JQ282065	JQ282020
	Atherinella marvalae	JQ282066	JQ282021
	Atherinella milleri	JQ282067	JQ282022
	Atherinomorus stipes	JQ282068	JQ282023
	Atherinopsis californiensis	JQ282063	JQ282018
	Atherinops affinis	JQ282061	no sequence
	Basilichthys semotilus	JQ282069	JQ282024
	Chirostoma consocium	JQ282070	JQ282025
	Chirostoma humboldtianum	JQ282071	JQ282026
	Chirostoma jordani	JQ282072	JQ282027
	Chirostoma labarcae	JQ282073	JQ282028
	Chirostoma patzcuaro	JQ282074	JQ282029
	Chirostoma riojai	JQ282075	JQ282030
	Labidesthes sicculus	JQ282077	JQ282031
	Leuresthes tenuis	JQ282078	JQ282032
	Melanorhinus microps	JQ282083	JQ282037
	Membras gilberti	JQ282080	JQ282034
	Membras martinica	JQ282081	JQ282035
	Menidia beryllina	JQ282079	JQ282033
	Menidia menidia	JQ282082	JQ282036
	Menidia penisulae	JQ282084	JQ282038
	Poblana ferdebueni	JQ282085	JQ282039
Bedotiidae	*Bedotia madagascariensis*	EF095640	no sequence
	Rheocles alaotrensis	JQ282058	no sequence
	Rheocles wrightae	JQ282059	no sequence
Melantoaeniidae	*Cairnsichthys rhombosomoides*	JQ282041	JQ282005
	Iriatherina werneri	JQ282047	JQ282006
	Melanotaenia australis	JQ282051	JQ282007
	Melanotaenia batanta	JQ282052	JQ282008
	Melanotaenia trifasciata	no sequence	NC_004385
	Rhadinocentrus ornatus	JQ282057	JQ282009
Notocheiridae	*Iso hawaiiensis*	no sequence	NC_01178
	Iso rhothophilus	JQ282048	JQ282010
	Iso sp.	JQ282049	JQ282011
	Notocheirus hubbsi	JQ282054	JQ282012
	Notocheirus hubbsi	JQ282055	JQ282013
Phallostethidae	*Neostethus bicornis*	JQ282053	no sequence
Pseudomugilidae	*Pseudomugil tenellus*	JQ282056	JQ282014
Telematherinidae	*Telmatherina antoniae*	JQ282060	JQ282015
Outgroups	*Ambassis* sp.	JQ282040	JQ282016
	Gambusia affinis	EF017411	NC_004388.1
	Hemiramphus brasiliensis	JQ282076	AF243865
	Mugil cephalus	EF095639	NC_003182
	Mugil curema	AY308783	EU715498
	Oryzias latipes	EF095641	NC_004387
	Poeciliopsis fasciata	EF017443	AF412149
	Scomberesox saurus	AY308771	AF243909
	Strongylura marina	JQ282086	AF243866
	Xiphophorus maculatus	EF017448	NC_011379

evolution used in the Bayesian analysis were chosen for each partition under the Akaike Information Criterion in the program JModelTest v0.1 (Posada, 2008). The Bayesian analysis, implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), consisted of four independent runs each using four chains sampling every 1000 generations for 20 million generations with all parameters unlinked. We assessed mixing of Metropolis coupled chains by confirming acceptance swap rates of adjacent chains fell between 10–70%. We also ensured standard deviation of split frequencies remained below 0.01 and potential scale reduction factors were 1.0. The first 4000 generations were discarded as burn-in, and posterior probabilities were determined by the frequency of clade occurrence in the remaining 16,000 trees.

We tested the alternative Atheriniform relationships of both Saeed et al. (1994) and Dyer and Chernoff (1996) by determining the posterior probability of these topologies. To do this, we created constrained trees in Mesquite v2.75 (Maddison and Maddison, 2011), loaded post-burn-in trees from our Bayesian analysis into PAUP and filtered the post burn-in trees to search for trees consistent with the constrained topology.

3. Results and discussion

3.1. Sequence data

The 1440 bp RAG1 data set consisted of 602 variable sites, of which 476 were parsimony informative. Our Cytb data set totaled 1141 bp, including 604 variable characters, 558 of which were parsimony informative. We detected no indels or gaps and inferred amino acid translations did not produce any stop codons in the dataset. The combined data matrix included 2581 characters, of which 1206 were variable sites and 1034 were parsimony informative. We recovered 19 equally parsimonious trees with a score of 7532 (CI = 0.269, RI = 0.538, RC = 0.144). Our ML analysis produced a well-resolved tree with a score of −33949.61621. All new sequences have been deposited in Genbank (Table 1).

3.2. Phylogenetic relationships of Atheriniformes

Our MP, ML, and BI resulted in nearly identical trees, differing mostly in levels of resolution and minor differences in relationships among Old World taxa, thus only the Bayesian tree (availabe on TreeBASE: http://purl.org/phylo/treebase/phylows/study/TB2:S12209) is shown and discussed (Fig. 2). Our results support Atheriniformes as a monophyletic group, a finding consistent with several recent studies (Dyer and Chernoff, 1996; Setiamarga et al., 2008; but see Sparks and Smith, 2004). We recovered Atherinopsidae as sister to all other Atheriniforms, all of which are Old World taxa with the exception of three species: *Atherinomorus stipes*, *Hypoatherina harriontonensis*, and *Alepidomus evermanni* (the latter not included this study). Thus, our results do not support Saeed et al.'s (1994) hypothesis that Old World Atherinids are sister to Cyprinodontiformes and Beloniformes, a finding consistent with recent studies (Dyer and Chernoff, 1996; Sparks and Smith, 2004; Setiamarga et al., 2008).

The most striking result of our study was that four of the seven families included in our study, Notocheiridae, Atherinopsidae, Atherinidae, and Melanotaeniidae were not monophlyetic. Our results suggest that Notocheiridae, as currently recognized, is a polyphyletic assemblage. Using Bayesian constraint searches we statistically reject the previous hypotheses of both Saeed et al. (1994) and Dyer and Chernoff (1996) ($p = 0.00$). Instead *Notocheirus hubbsi* was nested within the New World silverside family Atherinopsidae as the sister to the subfamily Menidiinae, while the Indo-Pacific genus *Iso* was sister to all other Old World Atheriniformes. The positions of *Notocheirus* and *Iso* were both well supported by posterior probabilities and ML and MP bootstrap values (1.0/100/100). Atherinopsidae was monophyletic aside from the inclusion of *Notocheirus*. For members of Atherinidae, we recovered *Hypoatherina* and *Atherinomorus* as sister to Melanotaenoidei and *Craterocephalus* and *Kestatherina* as sister to Bedotiidae but without

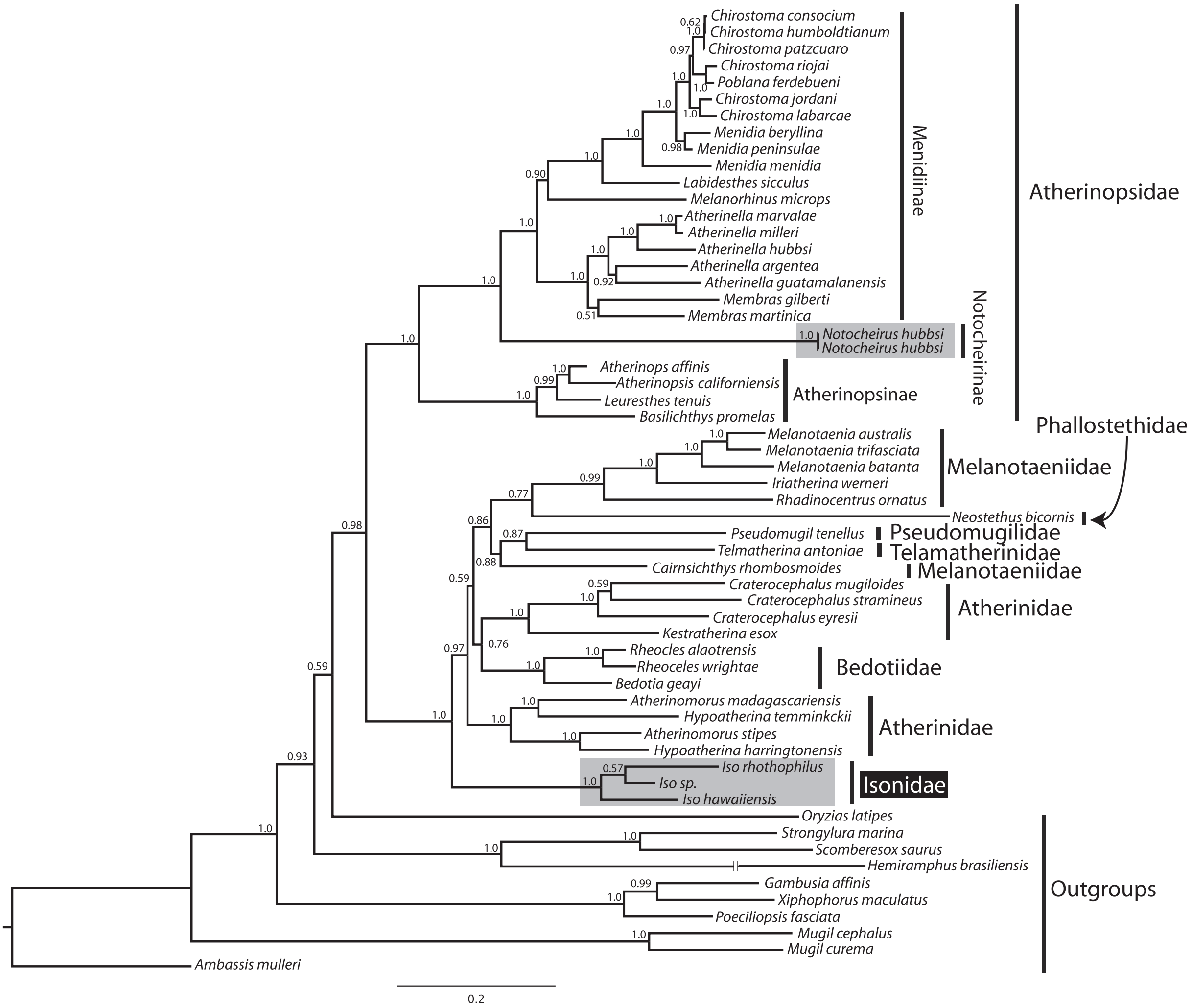

Fig. 2. Bayesian phylogeny for Atheriniformes based on concatenated RAG1 and CytB data set. Members of the previously recognized family Notocheridae are highlighted in gray boxes. We suggest resurrecting Isonidae (highlighted in black box) and including *Notocheirus hubbsi* in Atherinopsinae (see text for more details).

strong support (no support from MP bootstrap). Melanotaeniidae was monophyletic except for *Cairnsichthys*, which was sister to *Pseudomugil* (Pseudomugilidae), albeit with no statistical support. No study to date has suggested that Atherinidae or Melanotaeiniidae was not monophyletic, however, Sparks and Smith (2004) found only weak support for the monophyly of Atherinidae.

The phylogenetic position and affinities of members of Notocheiridae have long remained an unresolved question in Atheriniform systematics. Many of the morphological characters that were used to diagnose the monophyly of *Notocheirus* + *Iso* are functionally related to their habitat (e.g., body depth greatest at pectoral fin origin, ventral abdominal edge sharply keeled; Saeed et al., 1994; Dyer and Chernoff, 1996). In fact, the concept of Notocheiridae was originally proposed by Schultz (1948) based on body shape alone. Our data suggest that the overall similarity between *Iso* and *Notocheirus* is a result of convergent adaptation to a similar high-energy surf habitat, rather than due to phylogenetic relatedness. No previous study has included molecular data for both of these taxa, and using ecologically linked morphological traits to determine species groups can mislead phylogenetic inference due to convergence (e.g., Wiens et al., 2003). Thus, it is not surprising that resolving the phylogenetic placement of these enigmatic fishes has proven challenging using morphology alone (Parenti, 2005). Our study highlights the utility of molecular data for discovering instances of morphological convergence.

Previous Atheriniform topologies would have required several splits between New World and Old World lineages. Our phylogeny indicates an early split between New World and Old World silversides, with possibly a single dispersal to the New World explaining the presence of three atherinids in the Caribbean. Menidiinae is primarily tropical to sub-tropical in distribution, while Atherinopsinae has an anti-tropical distribution. The sister relationship between Menidiinae and *Notocheirus* presents an intriguing biogeographic pattern within Atherinopsidae, because *Notocheirus* is found in the temperate coastal waters of Chile and Argentina, a distribution that overlaps with species of *Odontesthes*, which are members of Atherinopsinae (Dyer, 1997, 1998, 2006). White (1986) suggested the anti-tropical distribution of Atherinopsinae resulted from rapidly increasing temperatures in lower latitudes during the Miocene. Presumably competition played a role in preventing menidiines from invading northward or southward, and likewise prevented Atherinopsinae lineages from re-invading the tropics. The phylogenetic placement of *Notocheirus* suggests this lineage overcame boundaries imposed by competition from atherinopsiines, and invaded the southern temperate waters. However, a robust test of White's hypothesis (1986) requires increased taxon sampling and a time-calibrated phylogeny to link diversification events in New World silversides to particular paleogeological events.

3.3. Taxonomic recommendations

The non-monophyletic taxonomic classifications indicated by our study can be rectified with several minor changes. We present our suggested taxonomic revisions in Fig. 2. Members of *Iso* previously formed the family Isonidae (reviewed by Dyer, 2006), which has continued to be recognized by some authors (Saeed et al., 2006). Based on our study, we support the placement of *Iso* in a family Isonidae. We also propose that *Notocheirus* should be included as a member of Atherinopsidae. *Notocheirus* is exceptional among Atheriniforms in lacking the first dorsal fin and epurals (both characters are present in *Iso*). This unusual morphology, combined with the phylogenetic position of *Notocheirus*, warrants the distinction of this genus from other Atherinopsids. Thus, we place *Notocheirus* in the subfamily Notocheirinae (sensu Schultz, 1950), and continue to recognize the subfamilies Menidiinae and Atherinopsinae (Chernoff, 1986; Dyer, 1997; Dyer and Chernoff, 1996). Addressing the composition and monophyly of Atherinidae and Melanotaeniidae requires more comprehensive taxonomic sampling and additional sequence data; thus, we refrain from any taxonomic suggestions regarding these taxa.

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