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A revised molecular phylogeny reveals polyphyly in *Schistura* (Teleostei: Cypriniformes: Nemacheilidae)

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Abstract

There is a general consensus that the genus *Schistura* (Nemacheilidae), currently with 241 species, is not monophyletic. However, weak morphological synapomorphies and a lack of genetic data for most species of *Schistura* and their presumptive relatives have prevented meaningful diagnoses of species groups within this genus. To aid in deciphering evolutionary relationships, sequence data from two mitochondrial genes (cytochrome *b* and D-loop) were implemented in phylogenetic analyses for species of *Schistura* and other nemacheilids for which data from earlier studies and recently collected material were available. This analysis of 67 nemacheilid species, including 28 species of *Schistura,* provides the most comprehensive phylogeny of Nemacheilidae to date. In the phylogenetic tree for the combined data set, species of *Schistura* clustered in three clades. One clade contained 14 species of *Schistura* and *Sectoria heterognathos* and was sister to *Homatula*. A second clade of 11 species of *Schistura* was in a larger clade with *Turcinoemacheilus kosswigi* and *Nemacheilus corica*. The third clade contained three species, all from the Mae Khlong basin of Thailand. Taxonomic implications of these results are discussed; however, a more taxon-rich dataset and nuclear sequence data are needed before making taxonomic changes.

Key words: cytochrome *b*, D-loop, loaches, *Nemacheilus*

Introduction

The family Nemacheilidae, known as the stone loaches, contains 720 species, of which 241 are assigned to the genus *Schistura* (Eschmeyer *et al*. 2018). Species of *Schistura* occupy most river drainages in mainland Asia from Pakistan to China while one species, *S. maculiceps* (Roberts 1989), occurs in the Sungai Pinoh (Kapuas River) on Borneo. Modern use of *Schistura* as the genus for this large and highly diverse group of Asian fishes dates back to several papers by Bănărescu & Nalbant (1966, 1968, 1974), as discussed by Mirza *et al*. (1981). There is a general consensus that the genus is not monophyletic (Kottelat 1990, Bănărescu & Nalbant 1995, Tang *et al*. 2006, Liu *et al*. 2012, Jamshidi *et al*. 2013), but the availability of limited morphological data and no genetic data for most species of *Schistura* and their presumptive relatives has prevented meaningful diagnoses of species groups as genera.

Genetic analyses to date include only a few species of *Schistura* in studies designed primarily to examine broad relationships among the Cobitoidea (Tang *et al*. 2006—4 species, Šlechtová *et al*. 2007—5 species, Liu *et al*. 2012—3 species), or the relationships within nemacheilid genera other than *Schistura* (Min *et al*. 2012—8 species, Jamshidi *et al*. 2013—5 species, Sember *et al*. 2015—7 species, Deng *et al*. 2016—13 species, Sayyadzadeh *et al*. 2016—13 species, Siva *et al*. 2017—12 species). Estimates of relationships among species of *Schistura* and their relationships to other nemacheilid genera in all of these studies, as in morphological studies, are constrained by the taxa included.

As a next step in deciphering evolutionary relationships among *Schistura*, sequence data from two mitochondrial genes (cytochrome *b* and D-loop) were used in phylogenetic analyses to compare species of

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Schistura and other nemacheilid genera using sequence data from earlier studies and recently collected material. This analysis of 67 nemacheilid species, including 28 species of *Schistura,* provides the most comprehensive phylogeny of Nemacheilidae to date.

Methods and materials

Molecular data analyzed included that for all species of *Schistura* available on GenBank with cyt *b* and D-loop sequences for which species identification could be verified with some certainty (Table 2). Species of other nemacheilid genera considered closely related to *Schistura* (Tang *et al*. 2006, Šlechtová *et al*. 2007) and original data on seven other species of *Schistura* were also included (Table 1). We did not include published data for specimens from aquarium dealers (e.g., Sember, *et al*. 2015) or from specimens for which no locality data were provided (e.g., Deng, *et al*. 2016, Siva *et al*. 2017). While many of these species were almost certainly correctly identified, it seems likely that some were not. Progress toward a more accurate assessment of relationships among species assigned to *Schistura* requires data with as much precision as possible.

Cyt *b* often is used in phylogenetic studies on cobitoids to elucidate intrageneric relationships (Tang *et al.* 2006, Min *et al.* 2012, Pandey *et al.* 2012, Sember *et al*. 2015, Deng *et al*. 2016). D-loop is not used as commonly but has been used to detect intraspecific variation and identify species complexes in *Lefua* and other cypriniforms (Liu *et al.* 2002, Sakai *et al.* 2003). The D-loop region is typically used in population studies because of its relatively rapid mutation rate (Liu *et al.* 2002, Sakai *et al.* 2003), making it useful for resolving nemacheilid lineages that diverged more recently than cyt *b* analysis can detect alone.

For the novel sequences, DNA extractions were accomplished using the protocol and equipment provided in a DNeasy Blood and Tissue Kit (Qiagen). Primers for the cytochrome *b* (L14724 and H15915) and D-loop (DL1 and DH2) regions were developed by Pandey *et al.* (2012) and Liu *et al.* (2002), respectively. The PCR products were sequenced in both forward and reverse directions using the PCR primers on a Beckman Coulter automated capillary sequencer. We were unable to obtain D-loop sequences for *S. dubia* or *S. geisleri*. Voucher specimens for novel sequences are at the Florida Museum of Natural History, University of Florida (UF). Novel sequences generated were deposited in GenBank (accession numbers MK301356–MK301366, see Table 1).

MUSCLE (Edgar 2004) was used to automatically align data from both genes, which were then adjusted by eye in Se-Al version 2.0a11 and compiled in Geneious (Kearse *et al.* 2012). MrModeltest version 2.2 (Nylander 2004), run in PAUP* 4.0 (Swofford 2002), was used to perform a priori nucleotide substitution model selection based on Akaike information criterion (AIC) (Table 3). Both gene fragments were concatenated and partitioned by gene and codon position. The genus *Triplophysa* was used as the rooted outgroup based on results of Tang *et al.* (2006) and Šlechtová *et al.* (2007). Phylogenetic reconstruction was performed using Bayesian Markov chain Monte Carlo (BMCMC) methods. Bayesian analysis was performed using MrBayes version 3.2.3 (Ronquist & Huelsenbeck 2003) in two independent runs with three heated and one cold chain in default MCMC settings for a total of 5.0×10^6 generations per run, with sampling trees and parameters every hundred generations. Tracer version 1.5.4 (Rambaut & Drummond 2009) was used to confirm stationarity, and the first 1.5×10^5 generations from each run were discarded as burn-in. TreeGraph 2 (Stöver & Müller 2010) was used to illustrate phylogenies.

Results

In the phylogenetic tree for the combined data set, species of *Schistura* occur in three separate clades (Fig. 1). Strong support exists for Clade 1, which is sister to *Homatula* and contains 14 species of *Schistura* and *Sectoria heterognathos*. Clade 2 contains 11 species of *Schistura* in a larger clade containing *Nemacheilus corica* and *Turcinoemacheilus kosswigi*. Relationships among these three genera are unresolved, and this clade is sister to *Oxynoemacheilus*. Strong support exists for Clade 3 containing three species of *Schistura*. This clade resides in a larger clade containing *Paracanthocobitis* plus Clade 2 of *Schistura* and its relatives without strong support for how these clades are related to one another. Clades 2 and 3 and their relatives are sister to a clade of *Nemacheilus*. This larger clade is sister to *Traccatichthys*, and this larger clade is sister to a clade of *Oreonectes* plus *Lefua*. This large clade is sister to Clade 1 of *Schistura* and *Homatula*, all of which then is sister to *Barbatula*.

The tree containing only cyt *b* data (Fig. 2) shows nearly the same overall relationships as the combined data tree. *Schistura* Clade 1, containing *Sectoria heterognathos*, is sister to *Homatula.* Clade 2 is monophyletic and in a polytomy with *Nemacheilus corica* and *Turcinoemacheilus kosswigi.* This group is sister to *Oxynoemacheilus,* and this larger group is sister to a group of three species of *Nemacheilus.* Clade 3 formed a monophyletic group in a polytomy containing Clade 2 and its relatives and *Paracanthocobitis*. The tree containing only D-loop data and only 11 species of *Schistura* (Fig. 3) shows the same relationships among the genera represented as the combined tree (though typically with stronger nodal support) except with fewer genera included, *Schistura* Clades 2 and 3 are sister taxa.

TABLE 1. Species for which sequence data were analyzed and their GenBank accession numbers. Names changed from those in GenBank in accordance with current nomenclature: *Paracanthocobitis mackenziei* (GQ478439) was labeled *Acanthocobitis botia* (see Singer & Page 2015); *Paracanthocobitis nigrolineata* (GQ174374, UF 172979) was labeled *Acanthocobitis zonalternans* (see Singer *et al*. 2017); *Schistura aurantiaca* (GQ174371, from UF 173049) was labeled *Schistura* sp. (see Plongsesthee *et al*. 2011); *S. magnifluvis* was misidentified as *S. bucculenta* (JN837654, KIZ 20080614); *S. mahnerti* (GQ174368, UF 173050) was misidentified as *S. desmotes*; *S. obliquofascia* was misidentified as *S. rupecula* (pers. comm., A. Barat; see Lokeshwor *et al*. 2012), and *S. vinciguerrae* was misidentified as *S. sikmaiensis* (JF340405, KIZ 2006010329). Species of *Homatula* were placed in *Paracobitis* in earlier studies.

Species	Cyt *b*	D-loop
Barbatula barbatula (Linnaeus)	AJ388467	DQ105327
Barbatula nuda (Bleeker)	DQ105252	EU670827
Barbatula sturanyi (Steindachner)	EF562769	-
Barbatula toni (Dybowski)	EF562772	EU670828
Barbatula vardarensis (Karaman)	EF562765	-
Homatula acuticephala (Zhou & He)	HM010530	-
Homatula anguillioides (Zhu & Wang)	HM010584	-
Homatula potanini (Günther)	DQ105203	NC_025321
Homatula variegata (Dabry de Thiersant)	AY625697	DQ105265
Lefua costata (Kessler)	EU670769	EU670826
Lefua echigonia Jordan & Richardson	AB080181	JQ410382
Lefua nikkonis (Jordan & Fowler)	AB100919	AB177660
Lefua pleskei (Herzenstein)	-	EU150380
Lefua sp. “Kii-Shikoku	-	AB177676
Lefua sp. “Tokai	-	AB251878
Nemacheilus corica (Hamilton)	GQ478442	-
Nemacheilus corica (Hamilton)	GQ174373	-
Nemacheilus masyae Smith	EF508597	-
Nemacheilus ornatus Kottelat	GQ174363	-
Nemacheilus pallidus Kottelat	GQ174370	-
Oreonectes platycephalus Günther	DQ105197	DQ105258
Oxynoemacheilus bureschi (Drensky)	GQ199476	-
Oxynoemacheilus merga (Krynicki)	EF562774	-
Oxynoemacheilus pindus (Economidis)	EF562773	-
Paracanthocobitis mackenziei (Chaudhuri)	GQ478439	-
Paracanthocobitis nigrolineata Singer, Pfeiffer & Page	GQ174374	-
Schistura amplizona Kottelat	JN837656	-
Schistura aurantiaca Plongsesthee, Page & Beamish	GQ174371	MK301356
Schistura balteata (Rendahl)	NC_008679	-
Schistura balteata (Rendahl)	MK301362	MK301357
Schistura beavani (Günther)	GQ478448	-
Schistura callichroma (Zhu & Wang)	JN837652	-

......*continued on the next page*

TABLE 1. (Continued)

Species	Cyt *b*	D-loop
Schistura caudofurca (Mai)	JN837651	-
Schistura cryptofasciata Chen, Kong & Yang	JF340401	-
Schistura desmotes (Fowler)	MK301363	MK301358
Schistura disparizona Zhou & Kottelat	JF340403	-
Schistura dubia Kottelat	MK301364	MK301359
Schistura fasciolata (Nichols & Pope)	DQ105201	EU697099
Schistura geisleri Kottelat	MK301365	
Schistura kloetzliae Kottelat	DQ105228	DQ105300
Schistura latifasciata (Zhu & Wang)	JN837653	-
Schistura longa (Zhu)	AY625698	DQ105261
Schistura macrotaenia (Yang)	JN837655	-
Schistura magnifluvis Kottelat	JN837654	-
Schistura mahnerti Kottelat	GQ174368	MK301360
Schistura montana McClelland	FJ711438	-
Schistura nicholsi (Smith)	DQ105202	DQ105264
Schistura obliquofascia Lokeshwor, *et al.*	HM636831	-
Schistura poculi (Smith)	JF340407	-
Schistura polytaenia (Zhu)	DQ105227	DQ105299
Schistura robertsi Kottelat	MK301366	MK301361
Schistura shuangjiangensis (Zhu & Wang)	JF340404	-
Schistura spilota (Fowler)	EF508596	-
Schistura tenebrosa Kangrang, Page & Beamish	JQ659026	-
Schistura vinciguerrae (Hora)	JF340405	-
Schistura vinciguerrae (Hora)	JF340406	-
Sectoria heterognathos (Chen)	DQ105200	DQ105262
Traccatichthys pulcher (Nichols & Pope)	DQ105198	AY091511
Triplophysa anterodorsalis Zhu & Cao	-	NC_024597
Triplophysa bleekeri (Sauvage & Dabry de Thiersant)	FJ406605	JQ680990
Triplophysa orientalis (Herzenstein)	DQ105251	DQ105323
Triplophysa robusta (Kessler)	-	NC_025632
Triplophysa rosa Chen & Yang	NC_019587	-
Triplophysa siluroides (Herzenstein)	EF212443	-
Triplophysa stenura (Herzenstein)	DQ105247	DQ105319
Triplophysa stewarti (Hora)	DQ105248	DQ105320
Triplophysa stolickai (Steindachner)	DQ105249	DQ105321
Triplophysa xiangxiensis (Yang, Yuan & Liao)	JN696407	JN696414
Turcinoemacheilus kosswigi “Bazoft” Bănărescu & Nalbant	GQ338826	-
Turcinoemacheilus kosswigi “Khoram” Bănărescu & Nalbant	GQ338827	-
Turcinoemacheilus kosswigi “Marboreh” Bănărescu & Nalbant	GQ338828	-

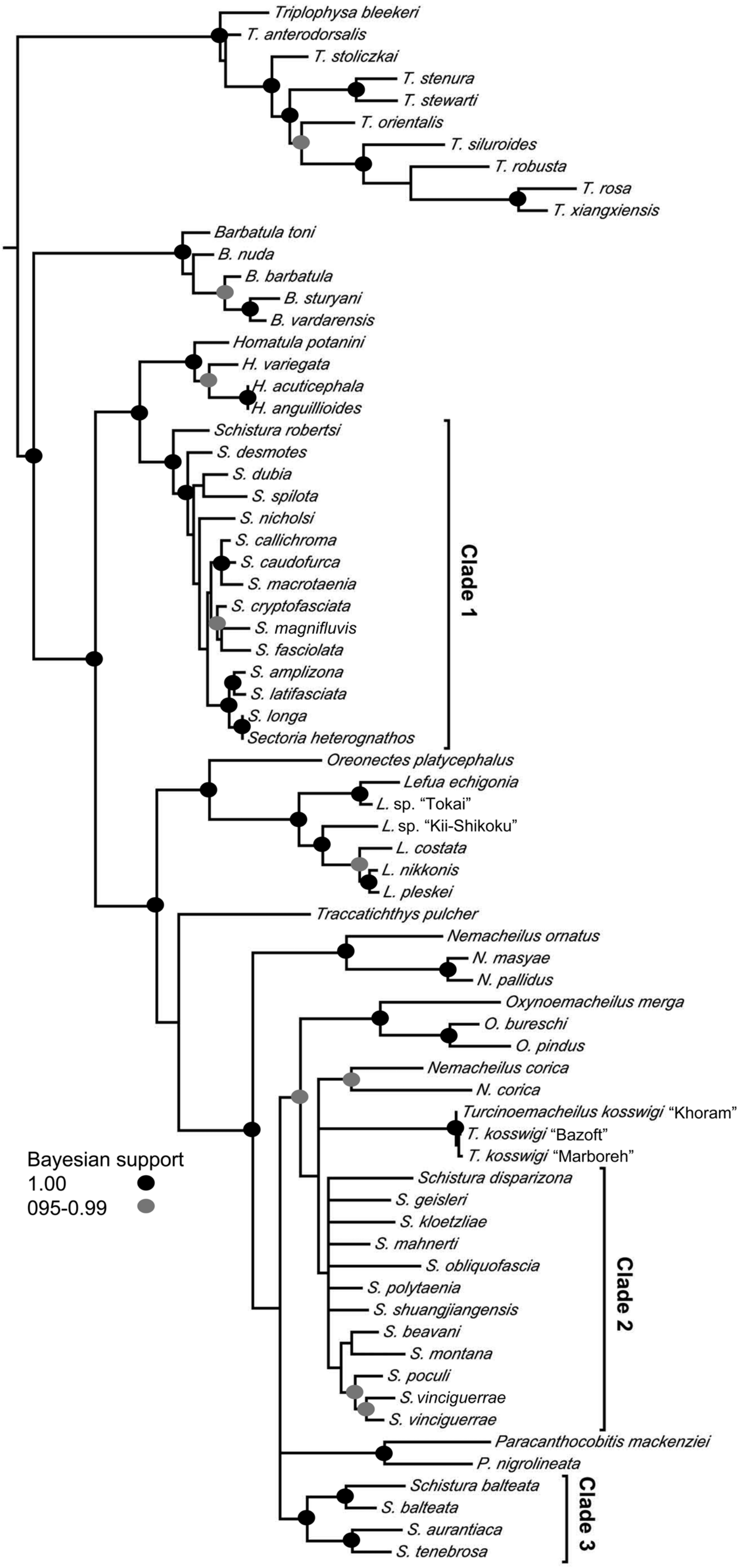

FIGURE 1. Bayesian consensus phylogeny depicting genus- and species-level relationships within the family Nemacheilidae. This tree was derived from a combined analysis of cytochrome *b* and D-loop gene fragments (1,116 bp and 776 bp, respectively).

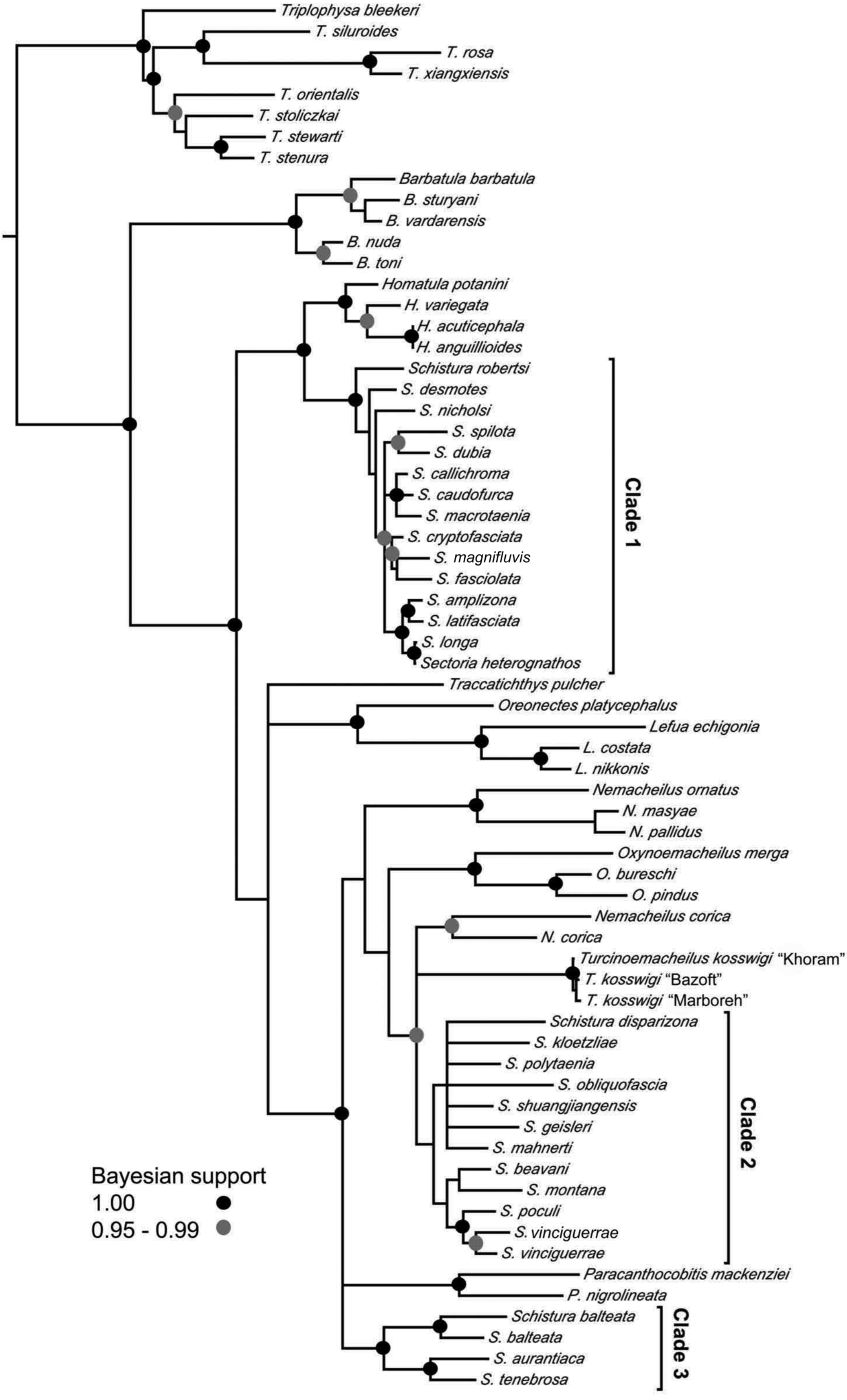

FIGURE 2. Phylogenetic estimate of relationships within the family Nemacheilidae generated with a 50% majority-rule consensus for the cytochrome *b* gene using MrBayes.

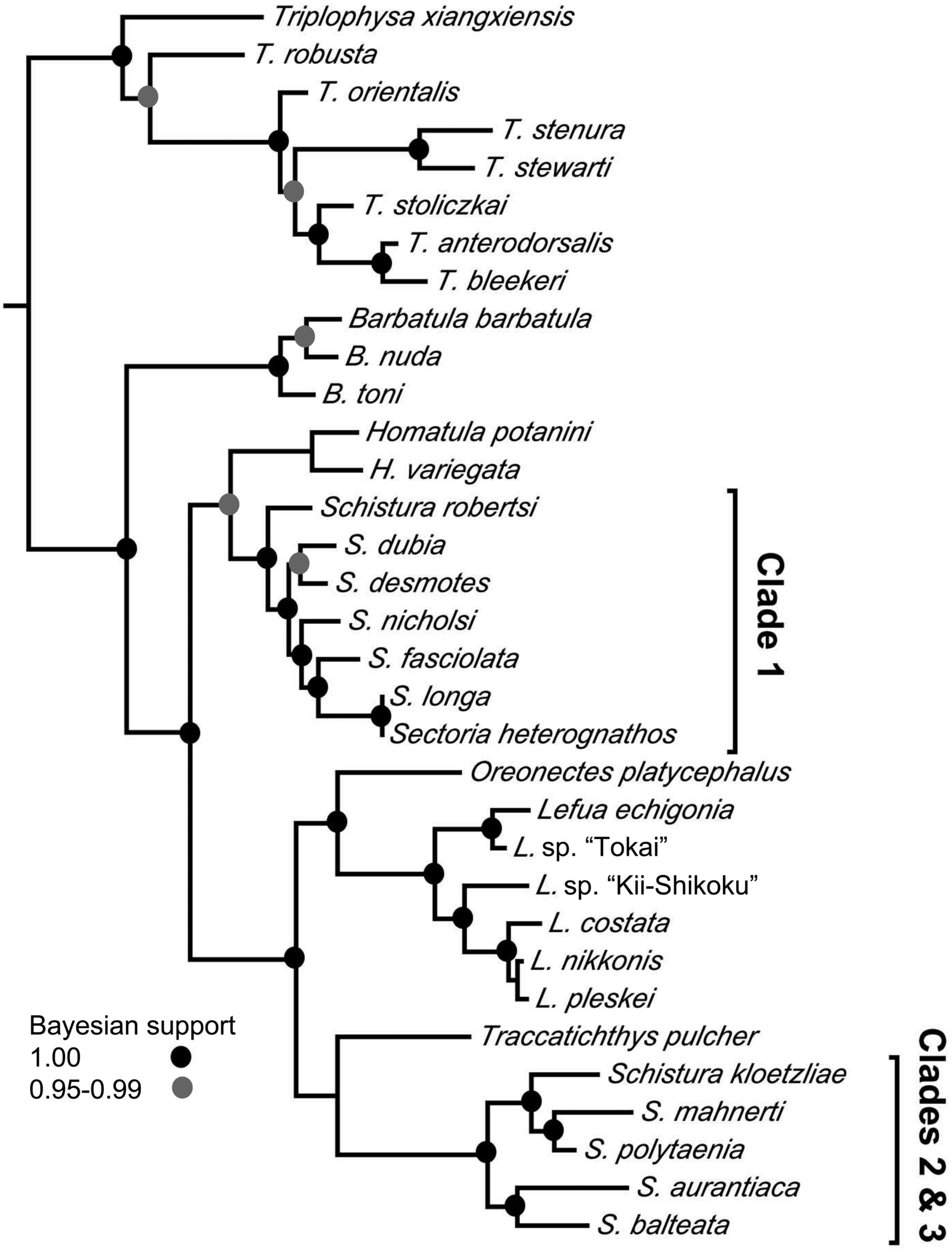

FIGURE 3. Phylogenetic estimate of relationships within the family Nemacheilidae generated with a 50% majority-rule consensus for the D-loop gene using MrBayes.

TABLE 2. Institutional catalog or voucher numbers, localities, and published references for *Schistura* sequence data included in this study.

Species	Catalog/voucher	River basin	Province	Country	Reference
S. amplizona	KIZ 2010003103	Mekong	Yunnan	China	Min *et al*., 2012
S. aurantiaca	UF 173049	MaeKhlong	Kanchanaburi	Thailand	This study
S. balteata	-	Aquarium	-	-	This study
S. balteata	-	-	-	-	Saitoh *et al*., 2006
S. beavani	-	Upper Ganges	-	India	Pandey *et al*., 2012
S. callichroma	KIZ 200401056	Red River	Yunnan	China	Min *et al*., 2012
S. caudofurca	KIZ 200401033	Red River	Yunnan	China	Min *et al*., 2012
S. cryptofasciata	KIZ 20050410011	Salween	Yunnan	China	Min *et al*., 2012
S. desmotes	UF183066	Chao Phraya	Chiang Mai	Thailand	This study
S. disparizona	KIZ 20050411040	Salween	Yunnan	China	-
S. dubia	UF 181122	Yom	Phrae	Thailand	This study
S. fasciolata	IHCAS0000047	-	Yunnan	China	Tang *et al*., 2006
S. geisleri	UF 181109	Wang	Lampang	Thailand	This study
S. kloetzliae	IHCAS0000016	-	Yunnan	China	Tang *et al*., 2006
S. latifasciata	KIZ 20050325004	Mekong	Yunnan	China	Min *et al*., 2012
S. longa	IHCAS0000050	-	Yunnan	China	Tang *et al*., 2006
S. macrotaenia	KIZ 20100035	-	Yunnan	China	Min *et al*., 2012
S. magnifluvis	KIZ 20080614	Mekong	Yunnan	China	Min *et al*., 2012
S. mahnerti	UF 173050	MaeKhlong	Kanchanaburi	Thailand	This study
S. montana	-	Khanda	-	India	Pandey *et al*., 2012
S. nicholsi	IHCAS0000047	-	-	-	Tang *et al*., 2006
S. obliquofasciata	-	-	-	India	Lokeshwor *et al*., 2012
S. poculi	KIZ 0310	-	Yunnan	China	-
S. polytaenia	-	-	Yunnan	China	Tang *et al*., 2006
S. robertsi	UF 185741	Tapi	Nakon Si Thammarat	Thailand	This study
S. shuangjiangensis	-	Mekong	Yunnan	China	-
S. spilota	-	Chao Phraya	Chiang Mai	Thailand	Šlechtová *et al*., 2008
S. tenebrosa	UF 181157	MaeKhlong	Kanchanaburi	Thailand	-
S. vinciguerrae	KIZ 2006010329	Irrawaddy	Yunnan	China	Tang *et al*., 2006
S. vinciguerrae	KIZ 200310725	Irrawaddy	-	-	Min *et al*., 2012

TABLE 3. Results from *a priori* model selection based on Akaike information criterion (AIC) conducted in MrModeltest 2.2 (Nylander 2004) for partitions of the dataset.

Loach phylogeny	Total characters	AIC model
Cyt *b* 1st position	372	SYM+I+G
Cyt *b* 2nd position	372	HKY+I+G
Cyt *b* 3rd position	372	GTR+G
D-loop	776	GTR+I+G

Discussion

This study is the most comprehensive analysis to date of relationships of species assigned to *Schistura*. Results strongly support the hypothesis that *Schistura* as presently conceived does not, as others have suggested (Kottelat 1990, Bănărescu & Nalbant 1995, Tang *et al*. 2006, Liu *et al*. 2012, Jamshidi *et al*. 2013), represent a monophyletic group of species. Some species of *Schistura* cluster more closely to *Homatula*, a group of 15 species in western China (Eschmeyer *et al*. 2018), than to other species of *Schistura*. Other species of *Schistura* cluster more closely to *Turcinoemacheilus* and *Oxynoemacheilus*, two genera found mainly in western Asia, and to *Nemacheilus* and *Paracanthocobitis*.

In the combined dataset (Fig. 1), Clade 1 includes 14 species of *Schistura* and *Sectoria heterognathos*. It consists mainly of species of *Schistura* (Fig. 4A, B) with 7–9 evenly spaced dark bars on the side of the body. *Sectoria heterognathos* is deeply embedded within this clade and closely related to *S. amplizona, S. latifasciata,* and *S. longa*. In the original description of *Sectoria* as a monotypic genus (Kottelat 1990) containing *S. atriceps* (Fig. 5), the genus was distinguished from *Schistura* and other nemacheilid genera primarily by the presence of horny sheaths with sharp edges on the lips and an intestine with numerous loops. Other characteristics mentioned are those that vary widely among nemacheilids (Kottelat 1990). Horny ridges on the lips and a multi-looped intestine are likely apomorphic states that evolved within Clade 1 of *Schistura*. Although these characteristics are sufficient to define a clade of two species (*Sectoria heterognathos* Chen, the second species of the genus, was described in 1999), recognition of *Sectoria* as a genus renders species of *Schistura* in Clade 1 paraphyletic. With additional data, it may be that all of the species in Clade 1 are recognized as species of *Sectoria*, or as *Acoura* Swainson 1839—seemingly the only other available genus-level name for species currently assigned to *Schistura* (Eschmeyer *et al*. 2018); however, more taxon-rich genetic and morphological investigations are required to determine synapomorphies for the clade.

Several of the species in Clade 2 in the combined dataset (Fig. 1) share a morphological apomorphy shown by *S. mahnerti* (Fig. 4C), the presence of dark bars on the side of the body that are widely spaced posteriorly and become narrower and more numerous anteriorly. However, also included in Clade 2 is *S. geisleri* (Fig. 4D), a species that is morphologically strikingly different from these species. Relationships within Clade 2 are mostly unresolved, although a close relationship is suggested among *S. poculi* and *S. vinciguerrae*. *Schistura shuangjiangensis*, embedded in Clade 2, was treated as a species of *Physoschistura* by Endruweit *et al*. (2016).

Clade 3 of *Schistura* includes *S. aurantiaca*, *S. balteata*, and *S. tenebrosa*. Samples for all of these species were collected in close proximity in the Mae Nam Khwae drainage of the Mae Khlong basin in western Thailand. These species may be closely related as indicated, or they may cluster as a result of past hybridization events that resulted in shared mitochondrial genotypes that subsequently differentiated only slightly. Mitochondrial introgression is common in some groups of fishes, e.g., Catostomidae (Unmack *et al*. 2014), and interspecific hybridization was suggested by Liu *et al.* (2012) as an explanation for some species relationships in loaches. The mitochondrial data suggest that *S. aurantiaca* (Fig. 4E) and *S. tenebrosa* are more closely related to one another than either is to *S. balteata* (Fig. 4F).

Nemacheilus is shown to be paraphyletic in our analysis. This is a relatively large genus with 32 species (Eschmeyer *et al.* 2018) of uncertain relationships. Like *Schistura*, the synapomorphies diagnosing the genus are limited, and more detailed studies of the genus using genetic and morphological data are needed. Only four species are included in our analysis, three of which form a strongly supported clade, and the fourth, *N. corica*, is more closely related to Clade 2 of *Schistura* plus *Turcinoemacheilus*. Unfortunately, the type species of *Nemacheilus, N. fasciatus*, described from Java, was not included in this study, and it is uncertain which of the two clades of *Nemacheilus* are more likely to represent the genus. However, others have suggested that *N. corica*, described from the Ganges River basin of India, is a morphologically divergent form that perhaps is not a species of *Nemacheilus* (e.g., Bohlen & Šlechtová 2013, Lokeshwor & Vishwanath 2014). *Pogonemacheilus* Fowler, 1937 is available (Eschmeyer *et al*. 2018) for the clade of species containing *Nemacheilus masyae*, although morphologically that clade appears more similar to *N. fasciatus* than does *N. corica.*

At least nine other studies of phylogenetic relationships among cobitoids based on genetic data have included three or more species of *Schistura*. Some provided little to no locality data, and because of uncertain identifications in several cases, discussion of the results of these studies are mostly limited to species included in our study.

Deng *et al*. (2016) analyzed cyt *b* sequences for nemacheilids, including 13 species of *Schistura*. Their results were partially similar to ours in that the species of *Schistura* divided into one clade of eight species, all members of our Clade 1, that formed the sister group to *Homatula*, and another clade of five species that clustered with *Nemacheilus*. Three of the five species in the clade sister to *Nemacheilus*, *S. poculi*, *S. shuangiangensis*, and *S.*

vinciguerrae (identified as *S. sikmaiensis*), were in our Clade 2, which was part of a large clade containing *Nemacheilus*. However, the other two species, *S. desmotes* and *S. longa*, were part of our Clade 1 and clustered with *Homatula*.

Šlechtová *et al*. (2007) included five species of *Schistura* in a broad analysis of relationships among families of Cobitoidea using *RAG1* sequence data. The five species of *Schistura* formed a monophyletic group that was sister to *Paracobitis variegatus* (now *Homatula variegata*—Kottelat 2012) and then clustered with *Triplophysa*. Two of the five species, *S. spilota* and *S. nicholsi*, were included in our analysis and were members of our Clade 1 and sister to *Homatula.*

Sember *et al*. (2015) produced a phylogeny of 19 species of nemacheilids including seven species of *Schistura,* based on cyt *b* and two nuclear genes (*RAG1* and *IRBP*). Their results were unusual in that only two of the species of *Schistura* clustered with one another; the other five species each clustered with species in another genus. The two species assigned to *Schistura* in Sember *et al*. that were included in our analysis were *S. fasciolata*, a member of our Clade 1, and *S. corica*, treated by us as *Nemacheilus corica*. *Nemacheilus corica* clustered with *Seminemacheilus*, a genus not included in our analysis.

Sayyadzadeh *et al*. (2016) produced a *COI* tree for nemacheilids that included 13 named and identified species of *Schistura* and several undescribed or unidentified species of *Schistura*. Only four of the species in their study overlapped with our study: *S. longa, S. poculi, S. vinciguerrae*, and *S. corica* (= *Nemacheilus corica*). *Schistura longa, S. poculi,* and *S. vinciguerrae* formed a strongly supported clade with *S. prolixifasciata* Zheng *et al.* 2012. Samples of *N. corica* were divided in two clusters, one with *Mesonoemacheilus guentheri* Day and one with *Schistura paucireticulata* Lokeshwor *et al.* 2013 (neither of which was in our analysis). Results differed from our analysis in that we had *S. poculi* and *S. vinciguerrae* in Clade 2 and sister to a clade containing *Nemacheilus*, but *S. longa* was in Clade 1 that clustered with *Homatula*. *Homatula* was not included in the Sayyadzadeh *et al*. analysis. Of particular interest in the Sayyadzadeh *et al*. study was the inclusion of *Acoura savona* (Hamilton 1822). *Acoura* has been treated recently as a synonym of *Schistura* (e.g., Kottelat 1990). In the analysis by Sayyadzadeh *et al*., *A. savona* was sister to a large clade including several genera of nemacheilids.

Siva *et al*. (2017) used the mitogenome of *Schistura reticulofasciata* (Singh & Bănărescu 1982) and mitochondrial data (not specified) downloaded from GenBank to generate a phylogeny of nemacheilids that included 12 species of *Schistura*. *Schistura kaysonei* Vidthayanon & Jaruthanin 2002, not included in our analysis, clustered with species of *Homatula*; the other 11 species formed a clade that was sister to all other nemacheilids in the analysis. Seven of the 11 species were included in our study in which *S. longa*, in Clade 1, clustered with *Homatula*, *S. geisleri* and *S. vinciguerrae* (identified as *S. sikmaiensis*) were members of Clade 2*,* and *S. balteata* (the basal group in the 11-species clade of Siva *et al*.) was in Clade 3. Siva *et al*. found *Nemacheilus corica* (as *S. corica*) to be a member of the *Schistura* clade, but basal to all species except *S. balteata*.

Tang *et al*. (2006) studied evolutionary rates in cyt *b* and the control region (mtDNA) in Cobitoidea and included four species of *Schistura* and *Sectoria heterognathos*. All of these species were included in our analysis (Tang *et al*. used the name *S. thai*, now considered a synonym of *S. nicholsi*). Results were similar to ours in that *S. kloetzliae*, in our Clade 2, clustered with three species of *Nemacheilus*, and *Sectoria heterognathos* clustered with the other three species of *Schistura*, all of which were in our Clade 1. This latter group was sister to two species of *Paracobitis*, both now in *Homatula* (Kottelat 2012).

Similarly, Jamshidi *et al*. (2013), mostly using data from Tang *et al*. (2006) in an analysis of cyt *b* data in a study directed at relationships of *Turcinoemacheilus,* included six species of *Schistura* (with *Nemacheilus corica* as *S. corica*) and *Sectoria heterognathos*. *Schistura beavani* (not in Tang *et al*.) and *S. kloetzliae*, in our Clade 2, clustered with three species of *Nemacheilus*. *Schistura fasciolata, S. nicholsi* (as *S. thai*)*, S. spilota,* and *Sectoria heterognathos*, all in our Clade 1, clustered with *Paracobitis* (=*Homatula*).

Liu *et al*. (2012) included three species of *Schistura* in a study of phylogenetic relationships of the Cobitoidea based on two mitochondrial and four nuclear genes. Two of the three species were included in our analysis. *Schistura fasciolata* in our Clade 1 paired with a species of *Paracobitis* (=*Homatula*), and *S. balteata* in our Clade 3 was sister to a clade including the other two species of *Schistura* and several genera of nemacheilids.

Min *et al*. (2012) included eight species of *Schistura* and *Triplophysa stenura* as outgroup taxa in a phylogenetic analysis of the genus *Homatula* using cyt *b* and *RAG1* sequence data. All eight species of *Schistura* were in our Clade 1 and formed a clade that clustered with *Homatula*.

FIGURE 4. Species of *Schistura* included in the phylogenetic analysis; all are from Thailand. Clade 1: (A) *S. desmotes,* UF 188065, 38.9 mm SL, Ping River, Chiang Mai Province; (B) *S. robertsi*, UF 185741, 50.8 mm SL, Tapee River, Nakhon Si Thammarat Province. Clade 2: (C) *S. mahnerti*, UF 188061, 71.9 mm SL, stream, Mae Khlong basin, Kanchanaburi Province; (D) *S. geisleri*, UF 191830, 28.0 mm SL, Wae Creek, Yan River basin, Surat Thani Province. Clade 3: (E) *S. aurantiaca*, UF 188063, 39.9 mm SL, Pracham Mai River, Kanchanaburi Province; (F) *S. balteata*, UF 191473, 52.0 mm SL, Pilok River, Kanchanaburi Province. Photographs by Zachary Randall and Jarred Randall.

FIGURE 5. *Sectoria atriceps*, UF 191745, 24.2 mm SL, Yang River, Nan Province, Thailand. Photograph by Zachary Randall and Jarred Randall.

Our study and this combination of studies, even with obvious shortcomings—including some uncertain identifications and the use of different taxa and genetic loci, identify taxonomic hypotheses that require further investigation. These include:

(1) Species of *Schistura* as currently recognized (Eschmeyer *et al*. 2018) are members of at least two large and apparently only distantly related groups within Nemacheilidae. One group (Clade 1 in our analysis) is more closely related to *Homatula.* The other group (Clade 2 in our analysis) appears to be relatively close to *Nemacheilus, Oxynoemacheilus, Paracanthocobitis,* and *Turcinoemacheilus*. Additionally, there may be another group (Clade 3) that appears closely related but not monophyletic with *Schistura* taxa from Clade 2. Although some studies discussed here have shown *Schistura* as monophyletic, they contained few species or too few genera to rigorously test the monophyly.

(2) *Sectoria*, represented by *S. heterognathos* in several studies, is a member of the clade containing species of *Schistura* related to *Homatula. Sectoria* is not a valid genus unless it is the name of a much larger group of species than currently recognized (represented by species in our Clade 1).

(3) *Acoura*, currently considered a synonym of *Schistura*, was distantly related to all species of *Schistura* and sister to a clade including several genera of nemacheilids in the analysis by Sayyadzadeh *et al*. (2016), suggesting that *Acoura* is a valid genus.

All of these hypotheses require further investigation with more genetic loci and more taxa, including representatives of more genera of nemacheilids, as well as more species of *Schistura* and data from the type species of *Acoura, Schistura,* and *Sectoria*. Future investigations must be based on data from specimens that have precise locality data and are deposited in institutional collections so that identifications can be verified. This is complicated by the fact that collecting specimens in many areas is difficult because of legal and cultural impediments. Some of these difficulties can be overcome by forming collaborations with colleagues from areas of particular geographic interest.

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