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Phylogenetic relationships of Acheilognathidae (Cypriniformes: Cyprinoidea) as revealed from evidence of both nuclear and mitochondrial gene sequence variation: Evidence for necessary taxonomic revision in the family and the identification of cryptic species



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ABSTRACT

Bitterlings are relatively small cypriniform species and extremely interesting evolutionarily due to their unusual reproductive behaviors and their coevolutionary relationships with freshwater mussels. As a group, they have attracted a great deal of attention in biological studies. Understanding the origin and evolution of their mating system demands a well-corroborated hypothesis of their evolutionary relationships. In this study, we provide the most comprehensive phylogenetic reconstruction of species relationships of the group based on partitioned maximum likelihood and Bayesian methods using DNA sequence variation of nuclear and mitochondrial genes on 41 species, several subspecies and three undescribed species. Our findings support the monophyly of the Acheilognathidae. Two of the three currently recognized genera are not monophyletic and the family can be subdivided into six clades. These clades are further regarded as genera based on both their phylogenetic relationships and a reappraisal of morphological characters. We present a revised classification for the Acheilognathidae with five genera/lineages: *Rhodeus*, *Acheilognathus* (new constitution), *Tanakia* (new constitution), *Paratanakia* gen. nov., and *Pseudorhodeus* gen. nov. and an unnamed clade containing five species currently referred to as "*Acheilognathus*". Gene trees of several bitterling species indicate that the taxa are not monophyletic. This result highlights a potentially dramatic underestimation of species diversity in this family. Using our new phylogenetic framework, we discuss the evolution of the Acheilognathidae relative to classification, taxonomy and biogeography.

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1. Introduction

Bitterlings are small fishes, typically less than 150 mm in length (Chen, 1998) and are native to Europe (three species) and east and southeast Asia (Kottelat and Freyhof, 2007). The species are associated with a variety of lowland freshwater habitats, including lakes, ponds, rivers and irrigation ditches. Aside from their striking nuptial coloration, bitterlings exhibit a remarkable breeding biology involving oviposition in the gill chambers of freshwater mussels (Unionidae and Margaritiferidae). Fertilization occurs in the mussel gill cavity and development is completed in the host mussel gill chamber (Smith et al., 2004). This breeding association makes species of bitterlings a focus of research aimed at understanding coevolutionary dynamics, life-history evolution, sexual selection, sperm competition, development and mate choice (Agbali et al., 2011; Casalini et al., 2009; Kitamura et al., 2012; Mills et al., 2005; Reichard et al., 2006, 2007, 2012; Spence and Smith, 2013; Spence et al., 2013).

Bitterlings are thought to form a monophyletic group and have traditionally been classified in the subfamily Acheilognathinae, one of the 11 subfamilies of Cyprinidae (Howes, 1991; Nelson, 2006). Recent molecular phylogenetic studies of cyprinid fishes have revealed significant findings regarding the relationships of the bitterling clade to other cyprinoids and a reclassification of Cyprinidae (Chen and Mayden, 2009; Mayden and Chen, 2010; Mayden et al., 2009; Saitoh et al., 2006; Tang et al., 2010, 2011; Yang, L. et al., 2012b). Current molecular-based analyses support the monophyly of Acheilognathinae as a subfamily within the Cyprinidae (Chen and Mayden, 2009; Tao et al., 2013; Saitoh et al., 2006; Wang et al., 2012b). However, Cyprinidae is paraphyletic with respect to Psilorhynchidae, as the latter family is nested within the former (Chen and Mayden, 2009; Mayden and Chen, 2010). The most closely related lineages to Acheilognathinae include: *Tanichthys* (mountain minnows), Tincinae (tench), Leuciscinae (minnows), and Gobioninae (gudgeons). These lineages are both temperate in distribution and include many species endemic to Eurasia and North America, and deeply nested within the “cyprinid” tree. Given the repeated recovery of monophyletic groups, congruent phylogenetic relationships, and the paraphyly of the “Cyprinidae”, Chen and Mayden (2009) argued for elevating the Acheilognathinae and some other previously recognized subfamilies of Cyprinidae to family status, within the superfamily Cyprinoidea.

1.1. Taxonomy

Bitterlings have a complicated taxonomic history. The Acheilognathidae currently includes about 74 species (from 117 available species names; Eschmeyer and Fong, 2014) and several undescribed species (Arai, 1988; Liu et al., 2006; Smith et al., 2004).

The classification inclusive of three genera, *Acheilognathus*, *Rhodeus*, and *Tanakia*, has been particularly unstable and has been retained largely as convention. Up to seven genera have been used for the group (*Acanthorhodeus*, *Acheilognathus*, *Rhodeops*, *Rhodeus*, *Paracheilognathus*, *Pseudoperilampus*, and *Tanakia*). Although classifications have included three genera some studies have not embraced this classification (Fujiwara et al., 2009; Hwang et al., 2014; Wang et al., 2012a). For instance, Arai and Akai (1988) used *Acheilognathus macropterus*; whereas Hwang et al. (2014) identified the species *Acanthorhodeus*. However, more recent studies (Duc et al., 2013; Li and Arai, 2010; Yang, Q. et al., 2010, 2011) generally agree in recognizing the “three genera scenario” (Arai and Akai, 1988). Diagnoses of the genera include characters related to karyotypes, color patterns on dorsal fins, and features of the lateralis system.

1.2. Previous hypotheses of acheilognathid phylogeny

Despite many molecular phylogenetic analyses of the Cyprinoidea (Chen and Mayden, 2009; Levin et al., 2012; Mayden et al., 2009; Perea et al., 2010; Tang et al., 2010, 2011; Tsigenopoulos et al., 2010; Yang, J. et al., 2012a; Yang, L. et al., 2012b), relationships within the Acheilognathidae have not been examined until very recently. Previous phylogenetic studies of bitterlings have been limited in character and/or taxon sampling (Bohlen et al., 2006; Chang et al., 2009; Kitamura et al., 2012; Yang, Q. et al., 2011; Zhu and Liu, 2006). Bohlen et al. (2006) proposed that *Rhodeus* in Europe was sister to the Asian species *Rhodeus sericeus*. However, their results failed to clarify whether the history of the multiple species in the genus involved one or more connections, or whether additional sister group relationships among European and Asian species need to be considered. Resolving this hypothesis of evolutionary and biogeographic events related to the origin of European bitterlings requires a greater sampling of species.

Prior to two recent studies (Cheng et al., 2014; Kawamura et al., 2014), the most comprehensive molecular-based study on species was that by Okazaki et al. (2001); however, this study suffered in data analysis. Okazaki et al. (2001) reconstructed relationships of bitterlings based only on partial sequences of 12S rRNA for 27 bitterling species/subspecies using Neighbor-Joining (NJ) analysis, a distance-based method of analysis that is known to be inadequate for the inference of species relationships. In their inferred NJ tree, *Acheilognathus* was recovered as monophyletic and sister to the weakly supported clade including *Rhodeus* and *Tanakia*. However, the monophyly of the latter two genera was not retained. Arai and Kato (2003) examined relationships using combined morphological and molecular (12S rRNA) characters as a follow up to a classification by Arai and Akai (1988). The former authors suggested a progressive evolution in bitterlings, implying a “trend” of bitterling evolution wherein *Tanakia* was the “ancestral” group, with both *Acheilognathus* and *Rhodeus* evolving from *Tanakia*. A more complete analysis by Chen and Mayden (2009), incorporating more taxa and characters in the cyprinoid phylogeny, contradicted these proposed evolutionary trends in bitterlings, and instead resolved *Acheilognathus* sister to other species.

The two most recent molecular studies on bitterlings by Cheng et al. (2014) and Kawamura et al. (2014) both increased taxonomic sampling (44 and 82 taxa included, respectively) relative to previous studies. However, both studies were limited in character sampling in using primarily cytochrome *b* sequences. Kawamura et al. (2014), while having increased taxonomic sampling, did not diversify species, with most coming from East Asia (particularly Korea and Japan) and only one sample was from Europe; no Middle Eastern species were included. Limited sampling from specific regions will tend to limit the resolution of evolutionary and biogeographic events.

Despite these limitations, the studies by Cheng et al. (2014) and Kawamura et al. (2014) consistently resolved Acheilognathidae as monophyletic with two major clades, *Acheilognathus* and *Tanakia-Rhodeus*. No previous studies (when multiple species from the three current genera were sampled) including the most recent ones have recovered *Tanakia* as monophyletic (Arai and Kato, 2003; Cheng et al., 2014; Kawamura et al., 2014; Okazaki et al., 2001). Thus, the monophyly of the genera *Tanakia* and *Rhodeus* remains questionable.

1.3. Objectives

In the present study we re-examined the phylogenetic relationships within Acheilognathidae using the largest molecular dataset assembled to date, with six nuclear gene loci (recombination activating gene 1 [*RAG1*], rhodopsin [*RH*], interphotoreceptor

retinoid-binding protein gene 2 [*IRBP2*], early growth response protein genes [*EGR* 1, 2B, and 3] (Chen et al., 2008) and one mitochondrial gene (cytochrome *b*, [*Cyt b*]) for 41 bitterling species represented by 117 individuals. These data and resulting analyses are the most comprehensive for this family and are capable of testing both previous phylogenetic hypotheses and hypotheses of a classification involving only three acheilognathid genera. The early evolution of Acheilognathidae is examined, providing new insight within the relationships among European and Asian species. Emphasis was also placed on greater geographic sampling of the most widely distributed species/subspecies (*A. macropterus*, *A. rhombus*, *A. tabira*, *A. barbatus*, *T. himantegus*, *R. ocellatus*, *R. sinensis*) to examine the possibility of undetected or cryptic species in the family.

2. Materials and methods

2.1. Sample collection

A total of 117 individuals of each bitterling species (41 species, 2 undescribed species, several subspecies) were sampled, where possible, across their native ranges. Specimens were sampled from natural habitats, commercial aquarists and known stocks from Japanese conservation research centers (e.g., *Tanakia tanago*). Following Eschmeyer's (2014) *Catalog of Fishes*, our sampling includes 50% of the listed species of *Acheilognathus*, 58% of *Rhodeus*, and 83% of *Tanakia*.

Most specimens were identified either by those providing samples or by the first author (CHC) using several identification resources, including information on local faunas, such as Chen et al. (1998) and Nakabo (2013). Species were identified as belonging to *Acheilognathus*, *Rhodeus*, or *Tanakia* following the classification of Arai and Akai (1988). Two small juvenile specimens could not be identified to either genus or species and are referred to as "Acheilognathidae gen. sp.". Similarly, it was not possible to identify some adult specimens to the level of species using current species diagnoses; these specimens may represent undescribed species and are identified with "sp.". Finally, for some specimens their characteristics were close to but did not completely fit the diagnosis of a described species; these examples are noted using the convention of "cf" (e.g., "*Acheilognathus*" cf. *striatus*).

Outgroup selection included multiple species and was based on previous phylogenetic hypotheses of Chen and Mayden (2009). Outgroup taxa included *Tinca tinca*, *Gobio gobio*, *Pelecus cultratus*, *Zacco sieboldii*, and *Danio dangila*, with the most distant outgroup being *Danio dangila*. Taxa and sample details are provided in Table 1.

2.2. DNA data collection

Genomic DNA was extracted either from fin or muscle preserved in 95% ethanol using the Quick Gene DNA tissue Kit S (Fujifilm, Tokyo, Japan). All primers of the six nuclear markers were from Chen et al. (2003, 2008), and López et al. (2004). Protocols for collecting DNA data from these markers follow those outlined in Chen et al. (2008). New pair-specific primers were designed for amplifying and sequencing *Cyt b*; these include *Cyt b*-F (5'-GAY TTG AAG AAC CAT CGT TGT A-3') and *Cyt b*-R (5'-CTT CGG ATT ACA AGA CCG ATG C-3'). PCR amplifications of *Cyt b* were performed in a mixture with a final volume of 25 μ L containing 10 ng template DNA, 25 μ mol of each pair of primers, 12.5 μ L of Fast-Run™ Advanced Taq Master Mix (ProTech, Taipei, Taiwan), and distilled water. Thermal cycling began with one cycle at 94 °C for 4 min; subsequently 35 cycles of denaturation at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and finally, a single

extension step at 72 °C for 5 min. PCR products were purified using a PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan). Sequencing was performed using ABI 3730 version 3.2 analyzer (Applied Biosystems), following protocols of ABI PRISM BigDye Sequencing Kit (PE Applied Biosystems, USA) and the same pairs of PCR primers (by Mission Biotech Inc., Taipei, Taiwan). All newly obtained sequences are available on GenBank; see Table 1 for accession numbers.

2.3. Sequence alignment and phylogenetic analysis

Genes were aligned manually using MEGA 5 (Tamura et al., 2011) and based on inferred amino acid translations. Sequenced bp for genes were as follows: *RAG1*, 1302 bp; *RH*, 801 bp; *IRBP2*, 807 bp; *EGR1*, 840 bp; *ERG2B*, 789 bp; *EGR3*, 843 bp; and *Cyt b*, 1140 bp. No indels were observed in the aligned sequences except for *EGR1* in which a deletion of three continuous nucleotides coding one amino acid occurred.

Phylogenetic analyses involved three operational datasets: (1) only mitochondrial-gene data set; (2) only nuclear-gene data set; and (3) a combined nuclear plus mitochondrial sequences. Analyses were performed using partitioned Maximum Likelihood (ML) and partitioned Bayesian approaches (BA). RAXML 7.0.4 (Stamatakis, 2006) was used for ML analyses (MLA). Partitions were set with respect to gene and codon position; the GTR + G + I model (with four discrete rate categories) was adopted for each partition. The ML tree was obtained by performing 100 different runs using the default algorithm of the program. The best ML tree was chosen from likelihood scores among suboptimal trees from each run. Nodal support for MLA was bootstrap analysis and determined using RAXML (Felsenstein, 1985); non-parametric bootstrap replications were 1000 with the ML criterion.

BA, as implemented in MR_{BAYES} 3.1.1 (Huelsenbeck and Ronquist, 2001), was used for the combined data set, involving 21 partitions based on gene and codon position. jModelTest (Posada, 2008) was used to select the best-fit model for each partition. Parameters for performing partitioned BA were as follows: "lset nst = 6" (for GTR model), "lset nst = 2" (for HKY model), "lset nst = 1" (for F81 model), "rates = invgamma" (G + I), "rates = propinv" (I), or "rates = gamma" (G), "unlink" (unlinking of model parameters across data partitions), and "prset ratepr = variable" (rate multiplier variable across data partitions). Two independent MCMC chains were conducted with 6,000,000 replicates, sampling one tree per 100 replications for each run. The distribution of log likelihood scores was examined to determine both stationarity for each search and the necessity for additional runs to reach convergence in log likelihoods. We discarded the initial trees with non-stationary log likelihood (as burn-in), and combined the remaining trees that resulted in convergent log likelihood scores from both independent searches. These trees were used to construct a 50% majority rule consensus tree. The values represented are *a posteriori* probabilities (PP) for BA. Nodal support was for BA trees was based on *a posteriori* probabilities (PP).

3. Results

A total of 6522 bp were aligned for the combined dataset of 117 bitterling and 5 outgroup taxa; Table 2 includes sequence length, number of variable sites, and number of parsimony informative sites for each genetic marker. Among sequences from the seven gene markers a deletion of one amino acid in *EGR1* was observed in only Acheilognathidae gen. sp., *A. typus*, *A. longipinnis*, *A. gracilis*, *A. asmussii*, *A. macropterus* (1) and (3), *A. macropterus* (5), *A. barbatus* (2), and *A. barbatulus* (8). However, with reference to the most likely

Table 1

List of bitterling species and their outgroup taxa sequenced for seven molecular markers (Cytochrome *b* [Cyt *b*], recombination activation gene 1 [RAG1], Rhodopsin [RH], interphotoreceptor retinoid-binding protein gene 2 [IRBP2], early growth response protein genes [EGR] 1, 2B, and 3) from the study with taxonomic identification, sample donor, location, individual number as utilized in Fig. 1 and GenBank accession numbers.

Genus	Species (individual no.)	Sample donor	Sample location	GenBank accession number								
				Cyt <i>b</i>	RAG1	RH	IRBP	EGR1	EGR2B	EGR3		
<i>Outgroup</i>												
	<i>Tinca tinca</i>			NC_008648	EU711162	FJ197070	FJ197121	FJ531280	FJ531309	FJ531338		
	<i>Gobio gobio</i>			NC_008662	EU292689	FJ197056	FJ197107	FJ531264	FJ531293	FJ531322		
	<i>Pelecus cultratus</i>			NC_008663	EU711144	FJ197045	FJ197095	FJ531272	FJ531301	FJ531330		
	<i>Zacco sieboldii</i>			NC_008653	EU292713	FJ197069	FJ197120	FJ531283	FJ531312	FJ531341		
	<i>Danio dangila</i>			NC_015525	EU292697	EU409660	EU409662	EU409724	EU409756	EU409788		
<i>Acheilognathidae</i>												
	<i>Acheilognathus asmussii</i>		Khanka Lake, Amur River basin	KF410698	KF471756	KF429359	KF434637	KF442283	KF444559	KF460155		
	<i>Acheilognathus barbatulus</i> (1)	Fan Li	Wuhan City, China	KF410707	KF471765	KF429368	KF434646	KF442292	KF444568	KF460164		
	<i>Acheilognathus barbatulus</i> (2)	Fan Li	Shanghai City, China	KF410699	KF471757	KF429360	KF434638	KF442284	KF444560	KF460156		
	<i>Acheilognathus barbatulus</i> (3)	Fan Li	Wuhan City, China	KF410700	KF471758	KF429361	KF434639	KF442285	KF444561	KF460157		
	" <i>Acheilognathus</i> " <i>barbatulus</i> (4)	Fan Li	Wuhan City, China	KF410701	KF471759	KF429362	KF434640	KF442286	KF444562	KF460158		
	<i>Acheilognathus barbatulus</i> (5)	Fan Li	Anhui Province, China	KF410706	KF471764	KF429367	KF434645	KF442291	KF444567	KF460163		
	<i>Acheilognathus barbatulus</i> (6)	Fan Li	Anhui Province, China	KF410702	KF471760	KF429363	KF434641	KF442287	KF444563	KF460159		
	<i>Acheilognathus barbatulus</i> (7)	Fan Li	Anhui Province, China	KF410703	KF471761	KF429364	KF434642	KF442288	KF444564	KF460160		
	<i>Acheilognathus barbatulus</i> (8)	Fan Li	Anhui Province, China	KF410704	KF471762	KF429365	KF434643	KF442289	KF444565	KF460161		
	<i>Acheilognathus barbatulus</i> (9)	Fan Li	Anhui Province, China	KF410705	KF471763	KF429366	KF434644	KF442290	KF444566	KF460162		
	<i>Acheilognathus barbatus</i> (1)	CToL ^a	Unrecorded	KF410711	KF471769	KF429372	KF434650	KF442296	KF444572	KF460168		
	<i>Acheilognathus barbatus</i> (2)	Fan Li	Anhui Province, China	KF410710	KF471768	KF429371	KF434649	KF442295	KF444571	KF460167		
	<i>Acheilognathus barbatus</i> (3)	Fan Li	Anhui Province, China	KF410708	KF471766	KF429369	KF434647	KF442293	KF444569	KF460165		
	<i>Acheilognathus barbatus</i> (4)	Fan Li	Anhui Province, China	KF410709	KF471767	KF429370	KF434648	KF442294	KF444570	KF460166		
	<i>Acheilognathus chankaensis</i> (1)	Fan Li	Anhui Province, China	KF410713	KF471771	KF429374	KF434652	KF442298	KF444574	KF460170		
	<i>Acheilognathus chankaensis</i> (2)	CToL ^a	Unrecorded	KF410712	KF471770	KF429373	KF434651	KF442297	KF444573	KF460169		
	<i>Acheilognathus cyanostigma</i>	CToL ^a	Unrecorded	KF410714	KF471772	KF429375	KF434653	KF442299	KF444575	KF460171		
	<i>Acheilognathus deignani</i>	Maurice Kottelat	Phongsali Province, Laos	KF410715	KF471773	KF429376	KF434654	KF442300	KF444576	KF460172		
	<i>Acheilognathus gracilis</i> (1)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410717	KF471775	KF429378	KF434656	KF442302	KF444578	KF460174		
	<i>Acheilognathus gracilis</i> (2)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410716	KF471774	KF429377	KF434655	KF442301	KF444577	KF460173		
	<i>Acheilognathus imberbis</i> (1)	Fan Li	Anhui Province, China	KF410718	KF471776	KF429379	KF434657	KF442303	KF444579	KF460175		
	<i>Acheilognathus imberbis</i> (2)	Fan Li	Anhui Province, China	KF410719	KF471777	KF429380	KF434658	KF442304	KF444580	KF460176		
	<i>Acheilognathus imberbis</i> (3)	Fan Li	Anhui Province, China	KF410720	KF471778	KF429381	KF434659	KF442305	KF444581	KF460177		
	<i>Acheilognathus intermedia</i>	Jae-Seong Lee	Korea	KF410721	KF471779	KF429382	KF434660	KF442306	KF444582	KF460178		
	<i>Acheilognathus longipinnis</i> (1)	Masaki Miya	Japan	KF410722	KF471780	KF429383	KF434661	KF442307	KF444583	KF460179		
	<i>Acheilognathus longipinnis</i> (2)	Masaki Miya	Japan	KF410723	KF471781	KF429384	KF434662	KF442308	KF444584	KF460180		
	<i>Acheilognathus macropterus</i> (1)	Feng Chen	Hubei Province, China	KF410728	KF471786	KF429389	KF434667	KF442313	KF444589	KF460185		
	<i>Acheilognathus macropterus</i> (2)	CToL ^a	Unrecorded	KF410726	KF471784	KF429387	KF434665	KF442311	KF444587	KF460183		
	<i>Acheilognathus macropterus</i> (3)	Takahiro Morosawa	Lake Kasumigaura, Japan	KF410727	KF471785	KF429388	KF434666	KF442312	KF444588	KF460184		
	<i>Acheilognathus macropterus</i> (4)	Chia-Hao Chang	Guangdong Province, China	KF410724	KF471782	KF429385	KF434663	KF442309	KF444585	KF460181		
	<i>Acheilognathus macropterus</i> (5)	Jie Li	Guangdong Province, China	KF410725	KF471783	KF429386	KF434664	KF442310	KF444586	KF460182		
	<i>Acheilognathus melanogaster</i> (1)	Takahiro Morosawa	Lake Kasumigaura, Japan	KF410730	KF471788	KF429391	KF434669	KF442315	KF444591	KF460187		
	<i>Acheilognathus melanogaster</i> (2)	Takahiro Morosawa	Lake Kasumigaura, Japan	KF410729	KF471787	KF429390	KF434668	KF442314	KF444590	KF460186		
	<i>Acheilognathus meridianus</i> (1)	Fan Li	Gvangsjih, China	KF410732	KF471790	KF429393	KF434671	KF442317	KF444593	KF460189		
	<i>Acheilognathus meridianus</i> (2)	Fan Li	Gvangsjih, China	KF410731	KF471789	KF429392	KF434670	KF442316	KF444592	KF460188		
	" <i>Acheilognathus</i> " sp.(1)	Fan Li	Anhui Province, China	KF410734	KF471792	KF429395	KF434673	KF442319	KF444595	KF460191		
	" <i>Acheilognathus</i> " sp. (2)	Fan Li	Anhui Province, China	KF410733	KF471791	KF429394	KF434672	KF442318	KF444594	KF460190		
	<i>Acheilognathus rhombeus</i> (1)	Takahiro Morosawa	Lake Kasumigaura, Japan	KF410738	KF471796	KF429399	KF434677	KF442323	KF444599	KF460195		
	<i>Acheilognathus rhombeus</i> (2)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410735	KF471793	KF429396	KF434674	KF442320	KF444596	KF460192		
	<i>Acheilognathus rhombeus</i> (3)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410736	KF471794	KF429397	KF434675	KF442321	KF444597	KF460193		
	<i>Acheilognathus rhombeus</i> (4)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410737	KF471795	KF429398	KF434676	KF442322	KF444598	KF460194		
	" <i>Acheilognathus</i> " cf. <i>striatus</i> (1)	Fan Li	Jiangxi Province, China	KF410740	KF471798	KF429401	KF434679	KF442325	KF444601	KF460197		
	" <i>Acheilognathus</i> " cf. <i>striatus</i> (2)	Fan Li	Jiangxi Province, China	KF410739	KF471797	KF429400	KF434678	KF442324	KF444600	KF460196		
	<i>Acheilognathus tabira tabira</i>	CToL ^a	Okayama, Japan	KF410741	KF471799	KF429402	KF434680	KF442326	KF444602	KF460198		
	<i>Acheilognathus tabira erythropterus</i>	Takahiro Morosawa	Lake Kasumigaura, Japan	KF410742	KF471800	KF429403	KF434681	KF442327	KF444603	KF460199		

(continued on next page)

Table 1 (continued)

Genus	Species (individual no.)	Sample donor	Sample location	GenBank accession number						
				Cyt <i>b</i>	RAG1	RH	IRBP	EGR1	EGR2B	EGR3
<i>Acheilognathus</i>	<i>tabira jordani</i>	CToL ^a	Shimane, Japan	KF410743	KF471801	KF429404	KF434682	KF442328	KF444604	KF460200
<i>Acheilognathus</i>	<i>tabira namakurae</i>	CToL ^a	Japan	KF410744	KF471802	KF429405	KF434683	KF442329	KF444605	KF460201
<i>Acheilognathus</i>	<i>tonkinensis</i>	Chia-Hao Chang	Aquarium shop, Taiwan	KF410745	KF471803	KF429406	KF434684	KF442330	KF444606	KF460202
<i>Acheilognathus</i>	<i>typus</i>	CToL ^a	Unrecorded	KF410746	KF471804	KF429407	KF434685	KF442331	KF444607	KF460203
<i>Acheilognathus</i>	<i>yamatsutae</i> (1)	Jae-Seong Lee	Korea	KF410748	KF471806	KF429409	KF434687	KF442333	KF444609	KF460205
<i>Acheilognathus</i>	<i>yamatsutae</i> (2)	CToL ^a	Korea	KF410747	KF471805	KF429408	KF434686	KF442332	KF444608	KF460204
<i>Acheilognathus</i>	<i>signifer</i> (1)	Jae-Seong Lee	Korea	KF410811	KF471869	KF429472	KF434750	KF442396	KF444672	KF460268
<i>Acheilognathus</i>	<i>signifer</i> (2)	Jae-Seong Lee	Jeollabuk-do, Korea	KF410810	KF471868	KF429471	KF434749	KF442395	KF444671	KF460267
<i>Acheilognathus</i>	<i>somjinensis</i>	Jae-Seong Lee	Korea	KF410812	KF471870	KF429473	KF434751	KF442397	KF444673	KF460269
<i>Rhodeus</i>	<i>amarus</i> (1)	Ján Koščo	Perin Village, near Kosice, Slovakia	KF410751	KF471809	KF429412	KF434690	KF442336	KF444612	KF460208
<i>Rhodeus</i>	<i>amarus</i> (2)	Jiří Musil	River Kyjovka (Danube Basin), Czech	KF410749	KF471807	KF429410	KF434688	KF442334	KF444610	KF460206
<i>Rhodeus</i>	<i>amarus</i> (3)	Alexandre Carpentier	Cher, France	KF410750	KF471808	KF429411	KF434689	KF442335	KF444611	KF460207
<i>Rhodeus</i>	<i>amarus</i> (E1)	Martin Reichard	River Oder, Poland	KF410752	KF471810	KF429413	KF434691	KF442337	KF444613	KF460209
<i>Rhodeus</i>	<i>amarus</i> (E2)	Martin Reichard	River Oder, Poland	KF410753	KF471811	KF429414	KF434692	KF442338	KF444614	KF460210
<i>Rhodeus</i>	<i>amarus</i> (W1)	Martin Reichard	River Kyjovka (Danube Basin), Czech	KF410754	KF471812	KF429415	KF434693	KF442339	KF444615	KF460211
<i>Rhodeus</i>	<i>amarus</i> (W2)	Martin Reichard	River Kyjovka (Danube Basin), Czech	KF410755	KF471813	KF429416	KF434694	KF442340	KF444616	KF460212
<i>Acheilognathidae</i>	gen. sp. (1)	Carl Smith	Khanka Lake, Amur River basin	KF410756	KF471814	KF429417	KF434695	KF442341	KF444617	KF460213
<i>Acheilognathidae</i>	gen. sp. (2)	Carl Smith	Khanka Lake, Amur River basin	KF410757	KF471815	KF429418	KF434696	KF442342	KF444618	KF460214
<i>Rhodeus</i>	<i>amurensis</i>	CToL ^a	Unrecorded	KF410758	KF471816	KF429419	KF434697	KF442343	KF444619	KF460215
<i>Rhodeus</i>	<i>atremius atremius</i>	CToL ^a	Fukuoka, Japan	KF410759	KF471817	KF429420	KF434698	KF442344	KF444620	KF460216
<i>Rhodeus</i>	<i>atremius suigensis</i> (1)	Kouichi Kawamura	Okayama, Japan	KF410760	KF471818	KF429421	KF434699	KF442345	KF444621	KF460217
<i>Rhodeus</i>	<i>atremius suigensis</i> (2)	Kouichi Kawamura	Okayama, Japan	KF410761	KF471819	KF429422	KF434700	KF442346	KF444622	KF460218
<i>Rhodeus</i>	<i>colchicus</i> (1)	Carl Smith	River Notanebi, Notabeni, Georgia	KF410762	KF471820	KF429423	KF434701	KF442347	KF444623	KF460219
<i>Rhodeus</i>	<i>colchicus</i> (2)	Carl Smith	River Notanebi, Notabeni, Georgia	KF410763	KF471821	KF429424	KF434702	KF442348	KF444624	KF460220
<i>Rhodeus</i>	<i>fangi</i>	Fan Li	Anhui Province, China	KF410764	KF471822	KF429425	KF434703	KF442349	KF444625	KF460221
<i>Rhodeus</i>	<i>meridionalis</i> (1)	Martin Reichard	River Vardar, Greece	KF410765	KF471823	KF429426	KF434704	KF442350	KF444626	KF460222
<i>Rhodeus</i>	<i>meridionalis</i> (2)	Martin Reichard	River Vardar, Greece	KF410766	KF471824	KF429427	KF434705	KF442351	KF444627	KF460223
<i>Rhodeus</i>	<i>notatus</i> (1)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410772	KF471830	KF429433	KF434711	KF442357	KF444633	KF460229
<i>Rhodeus</i>	<i>notatus</i> (2)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410767	KF471825	KF429428	KF434706	KF442352	KF444628	KF460224
<i>Rhodeus</i>	<i>notatus</i> (3)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410768	KF471826	KF429429	KF434707	KF442353	KF444629	KF460225
<i>Rhodeus</i>	<i>notatus</i> (4)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410769	KF471827	KF429430	KF434708	KF442354	KF444630	KF460226
<i>Rhodeus</i>	<i>notatus</i> (5)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410770	KF471828	KF429431	KF434709	KF442355	KF444631	KF460227
<i>Rhodeus</i>	<i>notatus</i> (6)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410771	KF471829	KF429432	KF434710	KF442356	KF444632	KF460228
<i>Rhodeus</i>	<i>ocellatus ocellatus</i> (1)	Chia-Hao Chang	Taiwan	KF410781	KF471839	KF429442	KF434720	KF442366	KF444642	KF460238
<i>Rhodeus</i>	<i>ocellatus ocellatus</i> (2)	Chia-Hao Chang	Sichuan Province, China	KF410773	KF471831	KF429434	KF434712	KF442358	KF444634	KF460230
<i>Rhodeus</i>	<i>ocellatus ocellatus</i> (3)	Jie Zhang	Beijing City, China	KF410774	KF471832	KF429435	KF434713	KF442359	KF444635	KF460231
<i>Rhodeus</i>	<i>ocellatus ocellatus</i> (4)	Xiu-Fa Hou	Guizhou Province, China	KF410775	KF471833	KF429436	KF434714	KF442360	KF444636	KF460232
<i>Rhodeus</i>	<i>ocellatus ocellatus</i> (5)	Fan Li	Shanghai City, China	KF410776	KF471834	KF429437	KF434715	KF442361	KF444637	KF460233
<i>Rhodeus</i>	<i>ocellatus kurumeus</i>	Tomoki Oonaka	Japan	KF410782	KF471840	KF429443	KF434721	KF442367	KF444643	KF460239
<i>Rhodeus</i>	<i>albomarginatus</i> (1)	Fan Li	Anhui Province, China	KF410780	KF471838	KF429441	KF434719	KF442365	KF444641	KF460237
<i>Rhodeus</i>	<i>albomarginatus</i> (2)	Fan Li	Anhui Province, China	KF410777	KF471835	KF429438	KF434716	KF442362	KF444638	KF460234
<i>Rhodeus</i>	<i>albomarginatus</i> (3)	Fan Li	Anhui Province, China	KF410778	KF471836	KF429439	KF434717	KF442363	KF444639	KF460235
<i>Rhodeus</i>	<i>albomarginatus</i> (4)	Fan Li	Anhui Province, China	KF410779	KF471837	KF429440	KF434718	KF442364	KF444640	KF460236
<i>Rhodeus</i>	<i>pseudosericeus</i> (1)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410783	KF471841	KF429444	KF434722	KF442368	KF444644	KF460240
<i>Rhodeus</i>	<i>pseudosericeus</i> (2)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410784	KF471842	KF429445	KF434723	KF442369	KF444645	KF460241
<i>Rhodeus</i>	<i>sericeus</i> (1)	Carl Smith	Lake Kenon, Amur River basin, East Russia	KF410785	KF471843	KF429446	KF434724	KF442370	KF444646	KF460242
<i>Rhodeus</i>	<i>sericeus</i> (2)	Carl Smith	Lake Kenon, Amur River basin, East Russia	KF410786	KF471844	KF429447	KF434725	KF442371	KF444647	KF460243
<i>Rhodeus</i>	<i>shitaiensis</i> (1)	Fan Li	Anhui Province, China	KF410787	KF471845	KF429448	KF434726	KF442372	KF444648	KF460244
<i>Rhodeus</i>	<i>shitaiensis</i> (2)	Fan Li	Anhui Province, China	KF410788	KF471846	KF429449	KF434727	KF442373	KF444649	KF460245
<i>Rhodeus</i>	<i>sinensis</i> (1)	Shun-Ping He	Wuhan City, China	KF410794	KF471852	KF429455	KF434733	KF442379	KF444655	KF460251
<i>Rhodeus</i>	<i>sinensis</i> (2)	Tomoki Oonaka	Aquarium shop, Japan	KF410793	KF471851	KF429454	KF434732	KF442378	KF444654	KF460250
<i>Rhodeus</i>	<i>sinensis</i> (3)	Fan Li	Shanghai City, China	KF410789	KF471847	KF429450	KF434728	KF442374	KF444650	KF460246
<i>Rhodeus</i>	<i>sinensis</i> (4)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410790	KF471848	KF429451	KF434729	KF442375	KF444651	KF460247
<i>Rhodeus</i>	<i>sinensis</i> (5)	Akimitsu Hanado	Gyeonggi-do, Korea	KF410791	KF471849	KF429452	KF434730	KF442376	KF444652	KF460248

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Table 2
Descriptive statistics of sequences for each gene locus used in this study.

Locus	Length (bp)	Number of variable sites	Number of parsimony informative sites
<i>Cyt b</i>	1140	560	505
<i>RAG1</i>	1302	369	245
<i>RH</i>	801	223	143
<i>IRBP2</i>	807	276	187
<i>FCR1</i>	840	215	103
<i>EGR2B</i>	789	141	68
<i>EGR3</i>	843	160	99

inferred phylogeny, the unique deletion appeared to have evolved independently eight times (see below, Fig. 1b).

All MLA and BA supported a monophyletic Acheilognathidae (see Fig. 1 and Fig. S1 in supplementary material). Topologies of mitochondrial (Fig. S1a) and nuclear (Fig. S1b) trees were largely congruent; the only observed discordance was in the position of *T. korensis* relative to some other species of *Tanakia* and *Acheilognathus*. In the mitochondrial gene tree, *T. korensis* was sister to a clade with polychotomous relationships; this clade included *Acheilognathus signifier*, *T. limbata* and *A. somjensis*. In the nuclear gene tree *T. korensis* was sister to *T. lanceolata* plus *A. intermedia* clade

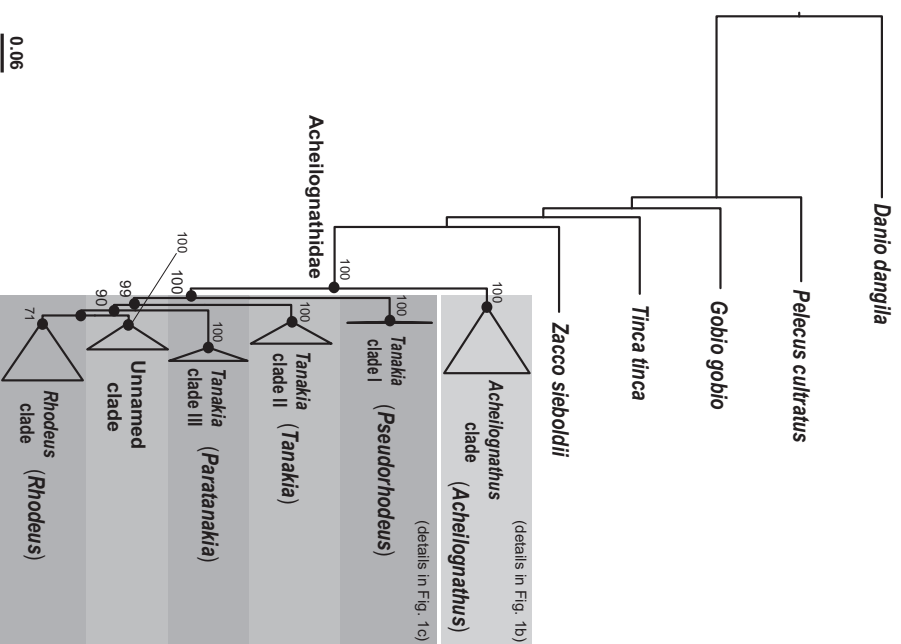


Fig. 1. Phylogenetic relationships of the Acheilognathidae from partitioned maximum likelihood analysis (21 partitions) and partitioned Bayesian analysis (21 partitions) of the combined dataset (7 genes: 6522 bp) (Parts A, B, and C). The topology from Bayesian inference is similar to ML tree; differences exist only on those relationships with weak statistical support. Numbers on branches are ML bootstrap values (those below 70% are not shown) and solid circles on branch nodes indicate statistically robust nodes with posterior probabilities from partitioned Bayesian analysis ≥ 0.95 .

<i>Rhodeus sinensis</i> (6)	Fan Li	Anhui Province, China	KF410792	KF471850	KF429453	KF434731	KF442377	KF444653	KF460249
<i>Rhodeus</i> sp. (1)	Carl Smith	Iran	KF410795	KF471853	KF429456	KF434734	KF442380	KF444656	KF460252
<i>Rhodeus</i> sp. (2)	Carl Smith	Iran	KF410796	KF471854	KF429457	KF434735	KF442381	KF444657	KF460253
<i>Rhodeus spinalis</i>	CToL ^a	China	KF410797	KF471855	KF429458	KF434736	KF442382	KF444658	KF460254
<i>Rhodeus suigenis</i>	Jae-Seong Lee	Korea	KF410798	KF471856	KF429459	KF434737	KF442383	KF444659	KF460255
<i>Tanakia himantegus chii</i> (1)	Chia-Hao Chang	Taiwan	KF410801	KF471859	KF429462	KF434740	KF442386	KF444662	KF460258
<i>Tanakia himantegus chii</i> (2)	Fan Li	Shanghai City, China	KF410799	KF471857	KF429460	KF434738	KF442384	KF444660	KF460256
<i>Tanakia himantegus chii</i> (3)	Fan Li	Zhejiang Province, China	KF410800	KF471858	KF429461	KF434739	KF442385	KF444661	KF460257
<i>Tanakia himantegus himantegus</i> (1)	Chia-Hao Chang	Taiwan	KF410804	KF471862	KF429465	KF434743	KF442389	KF444665	KF460261
<i>Tanakia himantegus himantegus</i> (2)	Chia-Hao Chang	Taiwan	KF410802	KF471860	KF429463	KF434741	KF442387	KF444663	KF460259
<i>Tanakia himantegus himantegus</i> (3)	Chia-Hao Chang	Taiwan	KF410803	KF471861	KF429464	KF434742	KF442388	KF444664	KF460260
<i>Tanakia korensis</i>	CToL ^a	Korea	KF410805	KF471863	KF429466	KF434744	KF442390	KF444666	KF460262
<i>Tanakia lanceolata</i> (1)	Tomoki Oonaka	Lake Kasumigaura, Japan	KF410807	KF471865	KF429468	KF434746	KF442392	KF444668	KF460264
<i>Tanakia lanceolata</i> (2)	Tomoki Oonaka	Gifu Province, Japan	KF410806	KF471864	KF429467	KF434745	KF442391	KF444667	KF460263
<i>Tanakia limbata</i> (1)	Tomoki Oonaka	Japan	KF410809	KF471867	KF429470	KF434748	KF442394	KF444670	KF460266
<i>Tanakia limbata</i> (2)	Tomoki Oonaka	Gifu, Japan	KF410808	KF471866	KF429469	KF434747	KF442393	KF444669	KF460265
<i>Tanakia tanago</i> (1)	Masaki Miya	Japan	KF410813	KF471871	KF429474	KF434752	KF442398	KF444674	KF460270
<i>Tanakia tanago</i> (2)	Masaki Miya	Japan	KF410814	KF471872	KF429475	KF434753	KF442399	KF444675	KF460271

^a CToL: Cypriniformes Tree of Life initiative.

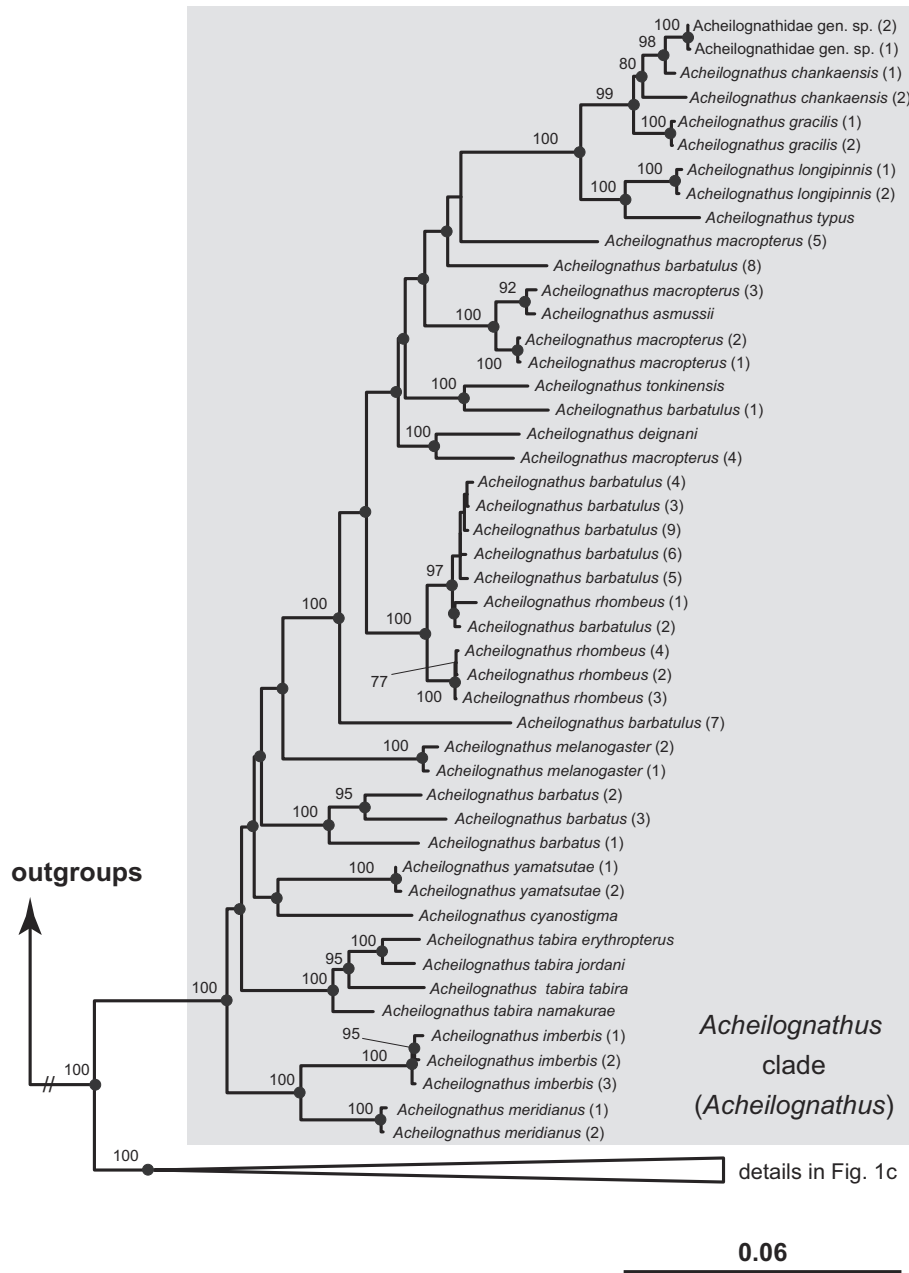


Fig. 1 (continued)

(Fig. S1).

Among the resulting phylogenies for any data set and analysis (Fig. 1), only *Rhodeus*, among the three currently recognized genera, was recovered as monophyletic (BS \geq 71%; PP \geq 0.95) (Fig. 1c). The Acheilognathidae was resolved as having two main reciprocally monophyletic groups, each with high support. The Acheilognathus clade contains most of species of *Acheilognathus* (Fig. 1b). The second clade includes all other species and was further subdivided into five clades: (1) a clade containing the remaining species of “*Acheilognathus*”, except for *A. intermedia*, *A. signifier*, and *A. somjinensis*, (2–4) three separate clades of species that are currently classified as *Tanakia* (clades I–III), and (5) the *Rhodeus* clade (Fig. 1c). *Tanakia* clade I included only *T. tanago*. *Tanakia* Clade II included only species from Japan and Korea, except for *T. tanago*, and three species formerly of *Acheilognathus* (*A. intermedia*, *A. signifier*, and *A. somjinensis*). Clade III included *Tanakia himantegus* from Taiwan and China.

Within *Acheilognathus sensu stricto* (*Acheilognathus* clade), relationships among the various main lineages were not well supported and internal branches are short relative to terminal branches. Such a pattern could reflect a rapid radiation occurring during an early diversification of *Acheilognathus* (Fig. 1b) or conserved anagenesis for the genes examined.

Within the second major acheilognathid group, the sister-group relationships among clades and within clades were generally well resolved and have high nodal support (Fig. 1a and c). The *Tanakia* clade I (or *T. tanago*) was the basal-most lineage, sister to the other four clades. The *Rhodeus* clade, while the more speciose group in this analysis, was deeply nested within acheilognathids (Fig. 1a and c).

Among the multiple individuals examined of *A. tabira*, *R. notatus*, *R. atremius*, *R. ocellatus*, and *T. himantegus*, the phylogenetic results of gene trees support their validity. However, intraspecific sequence divergence within these species was high, possibly indicating additional species diversity (e.g., cryptic species or

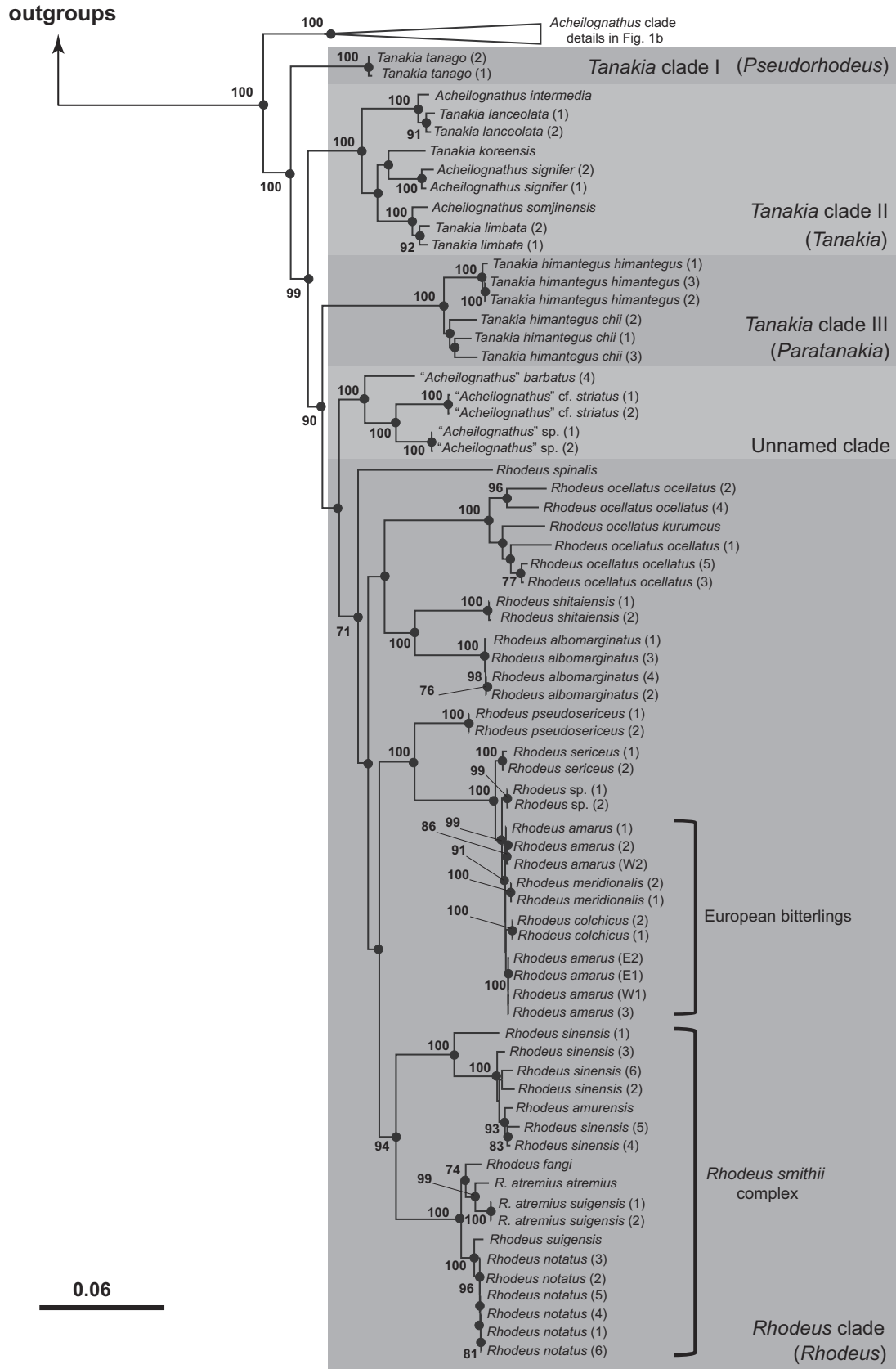


Fig. 1 (continued)

species complexes). Seven other species, representing about 18% of the species sampled in the study, where more than one specimen was examined (usually from different locations) did not group as

single gene-tree lineages by species. Gene lineages of *Acheilognathus chankaensis*, *A. rhombeus*, *R. sinensis*, and *R. amarus* were paraphyletic with respect to *Acheilognathid* gen. sp., *A. barbatus*,

R. amurensis, and *R. meridionalis* plus *R. colchicus*, respectively. Gene lineages of *A. macropterus*, *A. barbatulus*, and *A. barbatus* were resolved as polyphyletic groupings. Finally, “*Acheilognathus*” sp. and *Rhodeus* sp. are herein regarded as putatively undescribed species as they are morphologically distinct from other known species and both display unique phylogenetic positions.

4. Discussion

4.1. Phylogeny of the Acheilognathidae

Reciprocal monophyly of the three traditionally recognized acheilognathid genera has never been examined with large taxon and character sampling. Previous analyses had limitations in taxon and character sampling (Chen and Mayden, 2009; Fujiwara et al., 2009; Tang et al., 2011). The phylogeny of the Acheilognathidae based on mitochondrial 12S rRNA sequences, morphological, and karyological characters in earlier studies resolved *Tanakia* (inferred as monophyletic) as the sister group to *Acheilognathus* and *Rhodeus* (Arai, 1988; Arai and Kato, 2003). This result led Arai and Kato (2003) to hypothesize that *Tanakia* was the “ancestral” group, and both *Acheilognathus* and *Rhodeus* evolved from *Tanakia*. However, this hypothesis is inconsistent with results from two recent molecular studies based on either mitochondrial cytochrome *b* sequence data (Kawamura et al., 2014) or the combined data set with 12S rRNA sequences (Cheng et al., 2014). In these studies two reciprocal clades are resolved in the Acheilognathidae (*Acheilognathus* and *Tanakia-Rhodeus*).

Species relationships in the current study with a substantial increase in both taxa and characters, received high support. The resulting relationships are not consistent with the early hypothesis but are consistent with hypotheses of the two later studies (Cheng et al., 2014; Kawamura et al., 2014). The present analysis does not support the monophyly of *Tanakia* or *Acheilognathus*. While *Acheilognathus* was shown to be monophyletic in all previous molecular phylogenetic analyses (Cheng et al., 2014; Fujiwara et al., 2009; Kawamura et al., 2014; Okazaki et al., 2001; Yang, Q. et al., 2011; Zhu and Liu, 2006) the genus is not recovered as monophyletic. The *Acheilognathus* clade (or *Acheilognathus sensu stricto*) forms the sister group to remaining acheilognathid species (Fig. 1a).

The well-corroborated and consistent phylogenies resolved herein, based on two independent data sets, and the strong nodal support suggests that taxonomic revisions are warranted for the Acheilognathidae. Furthermore, given these relationships, diagnostic characters identified by Arai and Akai (1988) will have to be reexamined. Moreover, as the diploid chromosome numbers of the out-group taxa *Tinca tinca* and *Zacco* are 48 (Okazaki et al., 2001; Yu et al., 1987) this number used to group *Tanakia* ($2n = 48$) and *Rhodeus* ($2n = 48$ or 46) is not diagnostic. In comparison with previous studies (Cheng et al., 2014; Kawamura et al., 2014; Okazaki et al., 2001), this investigation is the first to provide evidence, with high statistical support, for the monophyly of *Rhodeus*, and the first to reveal the paraphyly of *Tanakia* (Fig. 1c). Given that the evolutionary history of a species is directly tied to its traits, previous studies drawing from earlier phylogenetic hypotheses as a framework for behavioral and biological evolution within acheilognathids should be reevaluated. Moreover, the present phylogenetic hypothesis also highlights several immediate taxonomic implications in need of attention, as well as highlighting opportunities to further test hypotheses regarding the evolution of bitterling species and their biology.

4.2. Taxonomic implications and revised classification

The Acheilognathidae includes six lineages, each being referred to separate genera. Only species of *Rhodeus* form a monophyletic

group, and species of “*Acheilognathus*” and “*Tanakia*” must be reallocated to newly proposed genera. The *Acheilognathus* clade, as recognized herein, retains its generic allocation given that it includes the type species, *A. melanogaster* (Kottelat, 2013). Arai and Akai (1988) indicate that this genus can be diagnosed morphologically from other acheilognathids, except for species of “*Acheilognathus*.” Species of *Acheilognathus* are diagnosed as having two transverse rows of white spots, equal in size, on the dorsal fin rays, with the upper row being narrower than the lower, a transverse row of black spots on the dorsal fin membranes, and serrations on pharyngeal teeth (versus teeth with vestigial serrations or less developed). Among the lineages of “*Tanakia*,” the genus name *Tanakia* applies to *Tanakia* Clade II, as it includes the type species, *Tanakia limbata* Jordan and Seale, 1906. Species of *Tanakia* Clade I and *Tanakia* Clade III can be diagnosed from species of *Tanakia* spp. of Clade II using the combination of morphological, genetic, and karyological characters. The details of the six genera including one retained, two revised, two new, and one undescribed genus are as follows:

Genus *Rhodeus*

Type species: *Rhodeus amarus* (Bloch, 1782)

Diagnosis: Diagnosed by Arai and Akai (1988) and retained herein. Species possessing a well-developed wing-like yolk sac projection, weakly developed and minute tubercles, and a diploid chromosome number 46.

Comments: While *Rhodeus smithii* was not included in this analysis, the other four species of this group form a monophyletic group, and we propose that the diagnostic characters of the *Rhodeus smithii* complex are synapomorphic.

Genus *Acheilognathus*

Synonyms: *Acanthorhodeus*, *Paracheilognathus*, and *Rhodeops* (Eschmeyer, 2014)

Type species: *Acheilognathus melanogaster* Bleeker, 1860

Diagnosis: *Acheilognathus* corresponds to our *Acheilognathus* clade (Fig. 1b). The diagnosis established by Arai and Akai (1988) is insufficient in distinguishing species of bitterlings in this clade from those in the unnamed clade. However, genetic data reveal that some molecular characters, in combination with characters provided by Arai and Akai (1988), can serve to diagnose this lineage. Additional morphological examination of species in this lineage is warranted to more fully develop a diagnosis of the lineage.

Comments: Whether the reduced diploid chromosome number of *Acheilognathus* ($2n = 44$ or 42) is shared with “*Acheilognathus*” from the unnamed clade is unknown and requires further karyological investigations of species of the latter lineage.

Genus *Tanakia*

Type species: *Tanakia limbata* (Temminck and Schlegel, 1846)

Diagnosis: Includes only species of *Tanakia* clade II (Fig. 1c). Diagnosis for the newly revised *Tanakia*, is derived from a modified diagnosis by Arai and Akai (1988). Lateral line complete, diploid chromosome number 48, and absence of 8M + 20SM + 18ST + 2A chromosomal constitution (Ojima et al., 1973; Sola et al., 2003; Ueda et al., 2001, 2006).

Comments: The three recently revised species of “*Acheilognathus*”: *A. intermedia*, *A. signifier*, and *A. somjinensis* (Yang, Q. et al., 2011) should be placed in *Tanakia* rather than *Acheilognathus*. Our findings confirm the taxonomic proposition by Arai and Akai (1988) to consider these three species as *Tanakia*. Thus, these species are transferred to *Tanakia*.

Genus *Pseudorhodeus* gen. nov. Chang, Chen, and Mayden

Type species: *Rhodeus tanago* Tanaka, 1909

Diagnosis: This new name applies to *Tanakia* clade I (Fig. 1c). Based on the description of *Pseudorhodeus tanago* (Tanaka, 1909) and karyological studies, this monotypic genus can be diagnosed from other “*Tanakia*” (Arai and Akai, 1988) in having an incomplete lateral line, a diploid chromosome number of 48, and a chromosomal constitution of 8M + 20SM + 20ST (Ojima et al., 1973).

Etymology: *Pseudorhodeus*. A noun in apposition. Latin *pseudo*, meaning false, and similarity to species of *Rhodeus*. [Tanaka \(1909\)](#) originally identified *Pseudorhodeus tanago* as a species of *Rhodeus*. The species was later transferred to *Tanakia* by [Okada \(1961\)](#). Most recently, it has been recognized in *Tanakia* ([Fricke, 2014](#)). Given that *P. tanago* was once viewed as *Rhodeus tanago*, and *P. tanago* and species of *Rhodeus* both possess an incomplete lateral line, *Pseudorhodeus* refers to the phenetic similarity of *P. tanago* being similar but not closely related to *Rhodeus*.

Genus *Paratanakia* gen. nov. Chang, Chen, and Mayden

Type species: *Acheilognathus himantegus* [Günther, 1868](#)

Diagnosis: This new name applies to *Tanakia* Clade III ([Fig. 1c](#)). Lineage distinguished from other species of “*Tanakia*” (including *Pseudorhodeus tanago*) using morphological and chromosomal characters identified in [Arai and Akai \(1988\)](#), [Chen et al. \(1998\)](#) and [Ueda et al. \(1997, 2006\)](#). Lateral line complete, diploid chromosome number 48, and chromosomal constitution 8M + 20SM + 18ST + 2A. Molecular bp characters also serve as synapomorphies for this genus at this time; nucleotide at the position 707 of the *RAG1* is A for this genus but is T for other acheilognathids, and bp composition at position 66 of the *EGR1* is C for this genus and A for other acheilognathids.

Etymology: *Paratanakia*. A noun in apposition. *Para*, from Greek word *parilis* meaning beside, near or by, and the genus name *Tanakia*, referring to the similarity to species of *Tanakia* “*himantegós*” is composed from *himás* and *himántos*. It’s a noun and the feminine form is equal. *Paratanakia himantegus* was placed in *Paracheilognathus* by [Günther \(1868\)](#), and then reclassified as *Tanakia* by [Arai and Akai \(1988\)](#). Currently, it is considered a member of *Tanakia* ([Eschmeyer, 2014](#)). Since *Paracheilognathus* is synonymous to *Acheilognathus* ([Arai and Akai, 1988](#)), *Paracheilognathus* is unavailable. *Paratanakia* derives from *Paratanakia himantegus* being morphologically more similar to the herein described monophyletic *Tanakia* (*Tanakia* Clade II) than to *Pseudorhodeus tanago* by having a complete lateral line.

The unnamed clade in ([Fig. 1c](#)) currently contains taxa inhabiting the Yangtze River basin. Species of “*Acheilognathus*” in the unnamed lineage (identified as unnamed clade, [Fig. 1c](#)) can only be diagnosed from *Acheilognathus* at this time using genetic characters. As the taxonomic sampling in our study included only 50% of the recognized species of *Acheilognathus* we refrain from naming this lineage at this time.

4.3. Species diversity and cryptic species

Taxonomy plays a crucial role in modern evolutionary biology ([Padial et al., 2009](#); [Smith et al., 2013](#); [Winsor, 2009](#)) with its primary focus being descriptions of new taxa, resolution of names that are valid, available, synonyms, and/or homonyms. Clarity in the classification of life with proper names applied to taxa is fundamental to studies of life forms ([Imamura and Nagao, 2011](#); [Iwatsuki and Heemstra, 2010](#); [Nielsen, 2011](#)).

Acheilognathidae currently includes about 74 valid species, yet around 120 species names are available and have been used ([Eschmeyer, 2014](#)). Species of this family continue to be discovered and described. Cryptic species are likely to exist within identified complexes and, based on gene trees, not all populations of currently recognized species are most closely related to one another. For example, gene trees of *A. rhombeus*, *T. koreensis* and *R. spinialis* are paraphyletic *sensu* [Kawamura et al. \(2014\)](#) and may indicate that additional species diversity exists within such groups. The phylogenetic framework established herein represents an initial step in the systematic revision of Acheilognathidae.

Among the species in the study from multiple geographic samples, gene trees suggest that seven do not group as would be expected of descendants from a most recent common ancestor.

This indicates that it is possible, given gene tree resolutions, multiple species exist within these seven species. These species include *A. chankaensis*, *A. rhombeus*, *R. sinensis*, *R. amarus*, *A. macropterus*, *A. barbatulus*, and *A. barbatus* ([Fig. 1b](#) and [c](#)). One of the most interesting instances is *A. macropterus*. Among the five individuals examined (from four different localities in China and Japan) they have resolved, with strong nodal support, into four independent lineages nested within the *Acheilognathus* ([Fig. 1b](#)). This may indicate that other species are currently masquerading under the name *A. macropterus*.

Acheilognathus asmussii (Lake Khanka, Amur River basin) is resolved as closely related to *A. macropterus* (3) from Lake Kasumigaura, Japan. This lineage is sister to another lineage including *A. macropterus* (1) and *A. macropterus* (2). *Acheilognathus macropterus* (4) from Guangdong Province in southern China is sister to *A. deignani* from the Mekong River in northern Laos ([Fig. 1b](#)). Finally, the phylogenetic position of *A. macropterus* (5), also collected from Guangdong Province in southern China, remains unresolved. Thus, the widely distributed species *A. macropterus* and *A. asmussii* may consist of at least four separate, geographically independent lineages, and possibly new species. Species delimitation requires additional sampling in other geographic areas and an examination of diagnostic traits. *Acheilognathus chankaensis* groups with acheilognathid gen. sp. with high statistical support, possibly indicating that the latter specimen is *A. chankaensis* or a close relative.

Currently available information indicates that the four specimens of *A. barbatus*, examined are nearly identical morphologically. However, these specimens group into two separate and distantly related lineages within the *Acheilognathus* clade ([Fig. 1b](#)) and unnamed clade ([Fig. 1c](#)). Cryptic species within *A. barbatus* may explain these divergent lineages under one name.

Gene trees of *A. rhombeus* resolve the species as an artificial grouping. *Acheilognathus rhombeus* (1) is nested within *A. barbatulus* ([Fig. 1b](#)), a result also revealed in analysis by [Kawamura et al. \(2014\)](#). Moreover, gene trees of *A. barbatulus* (7) resolve this specimen as an independent lineage relative to other *A. barbatulus* genetic lineages and *A. rhombeus* ([Fig. 1b](#)). Comparisons of nuclear and mitochondrial gene trees reveal no discordance in these relationships ([Fig. S1](#)). Thus, the possibility of mitochondrial introgression or inter-species hybridization is unlikely and additional species, yet to be discovered may exist within *A. rhombeus* and *A. barbatulus*.

Within *Rhodeus*, *R. sinensis* and *R. amurensis* are closely related. *Rhodeus sinensis* (1) is the most genetically divergent specimen found among samples ([Fig. 1c](#)). *Rhodeus amurensis* (Amur River drainage) is the sister-taxon to *R. sinensis* (4) and (5) from Korea. Notably, gene trees for specimens of *R. sinensis* (1, 3, 6), all sampled from the Yangtze River basin, revealed a paraphyletic grouping with respect to *R. amurensis* and the Korean sample of *R. sinensis*. A detailed morphological examination and taxonomic revision for these two species of *Rhodeus*, *R. albomarginatus* ([Li and Arai, 2014](#)), is morphologically most similar to *R. ocellatus*, but gene trees resolve the species as sister to *R. shitaensis*, an example illustrating that phenetic similarity does not necessarily reflect a genealogical relationship ([Fig. 1c](#)). Incongruence between the consistently resolved nuclear and mitochondrial gene trees and morphological similarity of samples of a particular species, may explain the above examples wherein gene trees do not support some specimens from the same or different populations of a species as sharing a common lineage.

The phylogenetic results presented here, based on multiple nuclear and mitochondrial gene sequences, indicate that some genetic groups of the individual species *A. chankaensis*, *A. macropterus*, *A. barbatulus*, *A. barbatus*, and *R. sinensis* ([Fig. 1b](#) and [c](#)), should

be carefully reexamined morphologically to investigate the existence of cryptic species and to better resolve the taxonomy of these species. Moreover, our phylogenetic results revealed that some taxa (“*Acheilognathus*” sp. and *Rhodeus* sp.) that appear as independent lineages are likely new species. *Acheilognathus tabira* (endemic to Japan) is represented by five subspecies based on differentiation in morphological characters, coloration, and their geographic distributions. Discrimination among these subspecies was also observed in genetic data (Arai et al., 2007; Kitamura et al., 2012). In our analysis of samples of four of the five subspecies (*A. t. tohokuensis* not sampled) gene trees corroborated their sharing of a well-supported common lineage. The resulting phylogeny presented herein is identical to that of Kitamura et al. (2012) based on *Cyt b*, *Glyt*, *Myh6*, *RAG1*, and *Ryr3* data. It is interesting that there is a substantial degree of genetic divergence among these subspecies derived from these two molecular studies. This finding implies a high nucleotide substitution rate for the gene markers used and, with further investigation, the diversification of possibly multiple new species (via taxonomic elevation from subspecies) currently within *A. tabira*.

4.4. Origin of European bitterling species

In the discovery and description of *Rhodeus colchicus* (a morphologically distinct European species) from the western Caucasus, Bogutskaya and Komlev (2001) evoked the assumption of putative reciprocal faunal exchange of bitterling species between Europe and East Asia. This conclusion was inferred from the fact that European *R. amarus* and East Asian *R. sericeus* have high-levels of morphological similarity (Bogutskaya and Komlev, 2001). This similarity led the authors to suggest two alternative colonization scenarios for Eurasian bitterlings; (1) speciation of *R. colchicus* and *R. sericeus* in East Asia and a subsequent parallel colonization of Europe by both lineages with subsequent extinction of the ancestral lineage of *R. colchicus* and (2) colonization of Europe by a common ancestor of *R. colchicus* and *R. sericeus* with subsequent split into *R. colchicus* and *R. sericeus* and a subsequent recolonization of eastern Asia by *R. sericeus*.

Bohlen et al. (2006) examined these hypotheses. They proposed species diversification and colonization of the Euro-Mediterranean area (*R. amarus*, *R. colchicus*, and *R. meridionalis*) occurred following their divergence of their most recent common ancestor (MRCA) from *R. sericeus* (East Asia), and a single dispersal event of the MRCA from East Asia to Europe. Results herein, incorporating *R. amarus* from the western and eastern lineages, *sensu* Bohlen et al. (2006), *R. colchicus*, *R. meridionalis*, and an undescribed Iranian species of *Rhodeus* (*Rhodeus* sp.) supports the hypothesis of Bohlen et al. (2006). The Iranian *Rhodeus* (not in Bohlen et al., 2006) is the sister-group to the monophyletic Euro-Mediterranean bitterling species, suggesting a possible broader and more continuous distribution of the MRCA of the Euro-Mediterranean and Iranian *Rhodeus*, extending from Europe through western Eurasia. The Korean species *Rhodeus pseudosericeus*, another species morphologically similar to *R. sericeus*, is the sister of the lineage including *R. sericeus*, *Rhodeus* from Iran, and the Euro-Mediterranean bitterlings. These relationships further corroborate the hypothesis that Euro-Mediterranean bitterlings are more recently evolved from *Rhodeus* and refute the hypothesis that *R. sericeus* (East Asia) is closely related to the morphologically similar *R. amarus* (European), providing another example of similarities in a simple context not reflecting genealogical relationships. The phylogenetic relationships among the Euro-Mediterranean species are not well resolved, possibly the result of relatively rapid speciation, incomplete lineage sorting and/or the gene flow among populations/species (Bryja et al., 2010). We have no conclusive evidence to identify which, if any, of these possible processes is the most.

5. Conclusions

Species of Acheilognathidae have emerged as a valuable model group in behavioral and evolutionary studies over the preceding two decades. We present a strongly supported phylogeny of this diversity in this family, including multiple species. This analysis represents the most comprehensive phylogenetic investigation of the family to date. Relationships are derived from 117 individuals from at least 41 species (some cryptic or undescribed species likely exist), exploiting nuclear and mitochondrial gene sequences, and evaluated using model-based analytical methods of evolutionary inference. Sister-group relationships in all trees generated are essentially identical and serve to reject the current classification for the Acheilognathidae, and provides evidence for several areas for further taxonomic investigation. This study also reveals additional species currently listed within a single species, which warrants additional investigation and possible reevaluation of the classification by Arai and Akai (1988). *Rhodeus* is monophyletic and its status is maintained. However, *Acheilognathus* and *Tanakia* are not monophyletic but represent multiple lineages in paraphyly. For phylogenetic and taxonomic consistency it is necessary to describe two new monotypic genera for two of the lineages—*Pseudorhodeus* and *Paratanakia*. An unnamed clade is recognized and discovery of this clade highlights the need for more species sampling and additional tests of its monophyly. Further taxonomic studies are recommended to aid in the identification of morphological or other traits useful in diagnosing lineages. Finally, the phylogenetic results strongly suggest that species diversity within the Acheilognathidae has been underestimated and warrants comprehensive revision.

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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.2014.08.026>.

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