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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

Chia-Hao Chang [a,b,c], Fan Li [d,e], Kwang-Tsao Shao [a], Yeong-Shin Lin [b,f], Takahiro Morosawa [g], Sungmin Kim [h], Hyeyoung Koo [i], Won Kim [h], Jae-Seong Lee [j], Shunping He [k], Carl Smith [l,m], Martin Reichard [m], Masaki Miya [n], Tetsuya Sado [n], Kazuhiko Uehara [o], Sébastien Lavoué [p], Wei-Jen Chen [p,*], Richard L. Mayden [c]

[a] Biodiversity Research Center, Academia Sinica, Taipei 11529, Taiwan
[b] Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 30068, Taiwan
[c] Department of Biology, Saint Louis University, St. Louis, MO 63103, USA
[d] Department of Oceanography, National Sun Yet-sen University, Kaohsiung 80424, Taiwan
[e] Institute of Biodiversity Science, Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, Fudan University, Shanghai 200433, China
[f] Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsinchu 30068, Taiwan
[g] Japan Wildlife Research Center, Tokyo 130-8606, Japan
[h] School of Biological Sciences, Seoul National University, Seoul 151-747, Republic of Korea
[i] Department of Biological Science, Sangji University, Wonju 220-702, Republic of Korea
[j] Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[k] Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
[l] School of Biology, University of St Andrews, St Andrews, Fife KY16 8LB, UK
[m] Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Květná 8, 603 65 Brno, Czech Republic
[n] Natural History Museum & Institute, Chiba 260-8682, Japan
[o] Aquatic Life Conservation Research Center, Research Institute of Environment, Agriculture and Fisheries, Osaka 572-0088, Japan
[p] Institute of Oceanography, National Taiwan University, Taipei 10617, Taiwan

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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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* Corresponding author at: Room 301, Institute of Oceanography, National Taiwan University, No. 1 Sec. 4 Roosevelt Road, Taipei 10617, Taiwan. Fax: +886 2 23637062.
E-mail address: wjchen.actinops@gmail.com (W.-J. Chen).

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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 *Acheiloghnathus 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 monphyletic 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