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Phylogenetic placements of the barbin genera *Discherodontus*, *Chagunius*, and *Hypselobarbus* in the subfamily Cyprininae (Teleostei: Cypriniformes) and their relationships with other barbins

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Abstract

The barbin genera *Discherodontus* Rainboth 1989, *Chagunius* Smith 1938 and *Hypselobarbus* Bleeker 1860 are distributed in Southeast and South Asia and are among the least studied taxa of the order Cypriniformes. Few morphological studies have been conducted on these genera and only a very limited number of morphological characters were employed to hypothesize or infer their monophyly, inter-relationships, and relationships with other barbins. The main aim of this study is to examine the monophyly of these three genera and propose hypothesis of relationship among these taxa and other barbins based on a molecular phylogeny of the subfamily Cyprininae. A total of 106 cypriniform species were sampled, including 64 species and 31 genera of barbins collected from Eurasia and Africa. Partitioned maximum likelihood analysis was performed using DNA sequences derived from five mitochondrial genes (5601 bp): cytochrome *c* oxidase subunit I (COI), cytochrome *b* (Cyt *b*), 16S ribosomal RNA (16S), NADH dehydrogenase subunits 4 (ND4) and subunits 5 (ND5). The resulting phylogeny demonstrates that, under current taxon sampling, *Discherodontus*, *Chagunius*, and *Hypselobarbus* are all monophyletic genera. Together they do not form a monophyletic group, as hypothesized in previous studies, but are instead part of three distinct and unrelated clades. *Discherodontus* constitutes the basal lineage of a clade formed by Southeast Asian barbins (e.g. *Poropuntius*, *Hypsibarbus*, *Balantiocheilos*); *Chagunius* is basal to a clade formed mainly by *Puntius* and allies (although this relationship was only weakly supported); *Hypselobarbus* and *Barbus carnaticus* formed a clade sister to a clade including *Tor*, *Neolissochilus*, *Labeobarbus*, and *Varicorhinus*. Homoplasy and shared plesiomorphy of some hypothesized important morphological characters employed in previous studies that led researchers to hypothesize earlier relationships are discussed.

Key words: homoplasy; mitochondrial; molecular; morphology; phylogeny

Introduction

With over 60 genera and 800 species, barbin fishes (e.g. *Barbus*, *Puntius*) have achieved great diversity in Eurasia and Africa. Historically, these taxa were thought to be members of the cypriniforme subfamily Cyprininae, a group that lack synapomorphic characters. Some recent molecular studies (e.g. Chen & Mayden 2009; Mayden & Chen 2010) have demonstrated that the Cyprininae is monophyletic and sister to the family Psilorhynchidae, rendering the current classification of subfamilies and families in the superorder Cyprinoidea of Cypriniformes somewhat chaotic. However, we continue to refer the target species to the Cyprininae, a group with which they consistently align with in molecular studies and the subfamily to which they have historically been allocated.

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The family Cyprinidae, as traditionally viewed, is the largest family of freshwater fishes of the world. Historical reasons for the continued recognition of the single family Cyprinidae are due to the lack of phylogenetic studies across the entirety of this diverse family (estimated number of species = 2420; Nelson 2006). In contrast, the Siluriformes, another group of ostariophysan fishes that is well studied and resolved for phylogenetic relationships contains about 2867 species and this diversity is spread over 35 families (Nelson 2006). This level of divisional classification not only creates order in an overly diverse group of organisms but also allows other scientists to work with taxa that are of "manageable" size when examining the internal relationships of larger clades or further resolution of species relationships. Phylogenetic resolution of large groups and the establishment of consistent classifications are the goals of the United States National Science Foundation Assembling the Tree of Life and Planetary Biodiversity Inventory programs and are the primary focus of our continuing research.

For convenience, the target group of this study is temporarily referred to the tribe Barbini herein. The tribe Barbini is essentially equivalent to the subfamily Barbinae of Chen *et al.* (1984) or the Barbin of Howes (1987). Some barbins are important food and sport fishes, e.g. Mahseer (*Tor*, *Neolissochilus*, *Naziritor*). There are also many species, e.g. genera *Puntius* and *Barbus*, that are extremely popular aquarium fishes. Earlier studies have worked towards understanding the phylogenetic relationships of species of *Barbus* and testing the monophyly of the genus, but many species of *Barbus* have never been included in any study, whether based on morphology or molecular data (e.g. Callejas & Ochando 2000; Tsigenopoulos *et al.* 2002a, 2002b, 2003; Zardoya & Doadrio 1998, 1999; Zardoya *et al.* 1999). Some studies investigated the interrelationships among species of *Barbus* and species of a few other cyprinine genera (e.g. Gilles *et al.* 1998, 2001; Durand *et al.* 2002; Levin *et al.* 2012). Other studies have examined the phylogenetic relationships among only a few or a significantly limited number of barbins (e.g. Cunha *et al.* 2002; Liu *et al.* 2002; Liu & Chen 2003; He *et al.* 2004; Xiao *et al.* 2005; He *et al.* 2008; Mayden *et al.* 2008, 2009; Nguyen *et al.* 2008; Turan 2008). The studies of Wang *et al.* (2007) and Li *et al.* (2008) did sample a number of barbins, but nearly all of the included species are endemic to China. Yang *et al.* (2010) sampled 24 barbin genera with most from Southeast Asia. Despite all the studies listed above, some barbin genera have never been sampled or have not been well represented in previous morphological or molecular phylogenetic studies.

The barbin genera *Discherodontus* Rainboth 1989, *Chagunius* Smith 1938 and *Hypselobarbus* Bleeker 1860 occur in Southeastern and Southern Asia. These genera are clearly among the least studied taxa of the Order Cypriniformes. *Discherodontus* are small fishes inhabiting upland rivers and streams of Southeast Asia and currently has four valid species (*D. ashmeadi* (type), *D. halei*, *D. parvus*, *D. schroederi*). They are distributed in Thailand, Cambodia, Laos, Malaysia, and China and exist in the Mekong, Chao Phraya, Maeklong, and Pahang drainages (Rainboth 1989, 1996; Doi 1997; Kottelat 1998; Shan *et al.* 2000). *Chagunius* includes three species (*C. chagunio* (type), *C. baileyi*, and *C. nicholsi*) and all are medium to large fishes inhabiting large upland rivers. They are known from the Ganges, Brahmaputra, Irrawaddy, Salween, and Sittang drainages of India, Bangladesh, Nepal, Myanmar, and Thailand (Rainboth 1986; Rahman 1989; Shrestha 1990; Talwar & Jhingran 1991; Doi 1997). Species of *Hypselobarbus* are medium to large barbins and the genus currently includes around 11 valid species; more species are likely present and we are currently studying diversity within this group. The current species diversity includes *Hypselobarbus mussullah* (type), *H. curmuca*, *H. dobsoni*, *H. dubius*, *H. kolus*, *H. kurali*, *H. lithopidos*, *H. micropogon*, *H. periyarensis*, *H. pulchellus*, *H. thomassi*. The genus is endemic to rivers of peninsular India (Talwar & Jhingran 1991; Jayaram 1999; Menon 1999).

Rainboth (1989) proposed that the *Discherodontus* was most closely related to *Chagunius*, and this group was also closely related to *Hypselobarbus*. He proposed that the group formed by these three genera is monophyletic. In the same paper, Rainboth suspected that these three genera are closely related to a group of Southeast Asian genera, his "Group A" that included *Albulichthys*, *Amblyrhynchichthys*, *Balantiocheilos*, *Cosmochilus*, *Cyclocheilichthys*, *Neobarynotus*, and *Puntioplites*. *Sikukia* was also suggested to be a possible member of this group. Rainboth (1989) further hypothesized that *Discherodontus* + *Chagunius* + *Hypselobarbus* were distantly related to genera of his "Group B," which contained *Acrossocheilus*, *Barbodes*, *Poropuntius*, and one undescribed genus, which was ultimately determined to be *Hypsibarbus* (Rainboth, pers. comm.). Those genera in "Group B" are the taxa into which species of *Discherodontus* have been previously classified. It should be noted, herein, that the name *Barbodes* has been used in the literature for a variety of different cyprinid fish species assemblages (Kottelat 1999). Southeast Asian species previously placed in *Barbodes* by Rainboth (1981, 1989, 1996), Kottelat (1989), and Kottelat *et al.* (1993) all currently belong to *Barbonymus* (Kottelat 1999). In Rainboth (1989), discussion as to the monophyly of the three genera *Discherodontus*, *Chagunius*, and *Hypselobarbus* and relationships among them and other barbin genera were mainly based on one or a few morphological characters. Monophyly of *Discherodontus* was hypothesized on the basis of the possession of two rows of pharyngeal teeth. Monophyly of *Chagunius* was hypothesized only on the basis of two characters: 1) possession of five unbranched dorsal-fin rays and 2) possession of four unbranched anal-fin rays. Monophyly of *Hypselobarbus* was hypothesized on the basis of one character, their shared possession of 9 branched dorsal-fin rays. Monophyly of the group formed by *Discherodontus*, *Chagunius*, and *Hypselobarbus* was hyothesized by their shared possession of prone gill rakers, and a black apex on the dorsal fin. Rainboth did not clearly state whether "Group A" is monophyletic or not, but none of the characters he listed are unique to taxa of this group. Rainboth (1989) further claimed that the monophyly of "Group B" was supported by their modified jaw morphology.

Rainboth (1981), an unpublished dissertation, classified *Discherodontus* (appeared as *Protopuntius*, a name never published) and *Chagunius* in his tribe Barbini, subtribe Barbi and placed *Hypselobarbus* (appeared as *Gonoproktopterus*, currently a junior synonym) in the tribe Babini, subtribe Tores. Later, Rainboth (1991) rearranged these three genera and placed them in the tribe Systomini, subtribe Poropuntii. Rainboth (1996) placed *Discherodontus* in his tribe Systomini, subtribe Osteobramae, but *Chagunius* and *Hypselobarbus* did not appear in the study. Neither Rainboth (1991) nor Rainboth (1996) provided any character support for these hypothesized classifications. Until now, Rainboth (1989) is the most extensive morphological study on *Discherodontus*, *Chagunius*, and *Hypselobarbus.* As far as we know, no molecular study has ever been conducted to explore the phylogenetic relationships of these three genera as well as their relationships with respect to other barbin genera.

The major objectives of the present study are two-fold: 1) to examine the phylogenetic relationships of *Discherodontus*, *Chagunius*, and *Hypselobarbus*; and 2) to explore the phylogenetic relationships among these genera relative to other barbins. Previous hypotheses proposed by Rainboth (1981, 1989, 1991, 1996) are discussed based on a phylogenetic tree built using DNA sequences derived from five mitochondrial genes.

Materials and Methods

Taxon Sampling. We sampled 106 cypriniform species. Among them, 88 species from 49 genera belong to the subfamily Cyprininae. A total of 64 species and 31 genera used in this study belong to the tribe Barbini (Table 1). As part of the investigation the ingroup included *Discherodontus ashmeadi* (type), *D. schroederi*, *Chagunius chagunio* (type), *C. baileyi*, *Hypselobarbus curmuca*, *H. kolus*, and *H. kurali*. Our ingroup analysis also included most of the genera in the proposed "Group A" and "Group B" *sensu* Rainboth (1989). For the seven genera in the "Group A", we included all the genera except *Neobarynotus*. The genus *Sikukia* was also included. All four genera in the "Group B" were sampled. Nine species of cyprinins, six oreinins, and nine labeonins were also included. Our analyses all have one or more representatives of the major cyprinine tribes and sub-tribes proposed by Rainboth (1981, 1991, 1996). Samples were collected from Southeast Asia, South Asia, East Asia, Europe, and Africa, all of which represent major distributional areas for the Cyprininae. Eighteen species were used as outgroups. Samples used in the current study were collected by the authors or obtained from collaborators in the Cypriniformes Tree of Life (CToL) and the All Cypriniformes PBI projects funded by USA NSF. Vouchered specimens were deposited at Saint Louis University and other institutions of our collaborators.

DNA extraction, PCR, and Sequencing. Whole genomic DNA was extracted from ethanol-preserved muscle or fin clips using DNeasy tissue extraction kits (Qiagen, USA). Five mitochondrial genes were amplified using PCR (Polymerase Chain Reaction). These include cytochrome *c* oxidase subunit I (COI), cytochrome *b* (Cyt *b*), 16S ribosomal RNA gene (16S), NADH dehydrogenase subunits 4 (ND4) and 5 (ND5). Primers and protocols used to amplify these genes follow Yang *et al*. (2010). No nuclear genes were employed in the present study because many cyprinines are polyploids (Arai, 2011) and usually have multiple alleles for each nuclear gene making it hard to establish homology. Amplified products were either directly purified using QIAquick PCR purification kits (Qiagen, USA) or gel purified using QIAquick gel extraction kits (Qiagen, USA). For DNA sequencing, we used the same primer sets as used for PCR amplifications. All sequencing was conducted at two facilities: htSEQ High-Throughput Genomics Unit (University of Washington, USA) and Macrogen (South Korea). All sequences generated from this study are deposited in GenBank and accession numbers for these and other sequences downloaded from GenBank are provided in Table 1.

TABLE 1. Taxa examined in this study with GenBank accession numbers for each gene

Taxa	**COI**	**Cyt *b***	**ND4**	**ND5**	**16S**
Order Cypriniformes					
Family Catostomidae					
Catostomus commersonii	AB127394	AB127394	AB127394	AB127394	AB127394
Family Gyrinocheilidae					
Gyrinocheilus aymonieri	AB242164	AB242164	AB242164	AB242164	AB242164
Family Cobitidae					
Cobitis striata	AB054125	AB054125	AB054125	AB054125	AB054125
Family Balitoridae					
Homaloptera leonardi	AB242165	AB242165	AB242165	AB242165	AB242165
Family Cyprinidae					
Subfamily Acheilognathinae					
Acheilognathus typus	AB239602	AB239602	AB239602	AB239602	AB239602
Rhodeus ocellatus	AB070205	AB070205	AB070205	AB070205	AB070205
Subfamily Gobioninae					
Gobio gobio	AB239596	AB239596	AB239596	AB239596	AB239596
Hemibarbus labeo	AB070241	AB070241	AB070241	AB070241	AB070241
Subfamily Leuciscinae					
Alburnus alburnus	AB239593	AB239593	AB239593	AB239593	AB239593
Ctenopharyngodon idella	EU391390	EU391390	EU391390	EU391390	EU391390
Notropis stramineus	DQ536429	DQ536429	DQ536429	DQ536429	DQ536429
Subfamily Phoxininae					
Rhynchocypris perenurus	AP009061	AP009061	AP009061	AP009061	AP009061
Subfamily Xenocyprinae					
Xenocypris macrolepis	AP009059	AP009059	AP009059	AP009059	AP009059
Subfamily Cultrinae					
Ischikauia steenackeri	AB239601	AB239601	AB239601	AB239601	AB239601
Subfamily Danioninae					
Danio rerio	AC024175	AC024175	AC024175	AC024175	AC024175
Esomus metallicus	AB239594	AB239594	AB239594	AB239594	AB239594
Incertae sedis					
Nipponocypris sieboldii	AB218898	AB218898	AB218898	AB218898	AB218898
Opsariichthys uncirostris	AB218897	AB218897	AB218897	AB218897	AB218897
Subfamily Cyprininae					
Tribe Barbini					
Chagunius chagunio	JX066746	JX066764	JX066777	JX066790	JX066730
Chagunius baileyi	JX066747	JX066765	JX066778	JX066791	JX066731
Discherodontus ashmeadi	JX066748	JX066766	JX066779	JX066792	JX066732

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TABLE 1. (Continued)

Taxa	COI	Cyt *b*	ND4	ND5	16S
Discherodontus schroederi	JX066749	JX066767	JX066780	JX066793	JX066733
Hypselobarbus curmuca	HM010708	HM010720	HM010732	HM010732	HM010696
Hypselobarbus kolus	HM010711	HM010723	HM010735	HM010735	HM010699
Hypselobarbus kurali	HM010716	HM010728	HM010740	HM010740	HM010704
“Group A”					
Albulichthys albuloides	JX066750	JX066768	JX066781	JX066794	JX066734
Amblyrhynchichthys truncatus	JX066751	JX066769	JX066782	JX066795	JX066735
Balantiocheilos melanopterus	AP011243	AP011243	AP011243	AP011243	AP011243
Cosmochilus harmandi	JX066752	JX066770	JX066783	JX066796	JX066736
Cyclocheilichthys armatus	HM536926	HM536827	HM536745	HM536861	HM536784
Cyclocheilichthys janthochir	HM536907	HM536808	HM536727	HM536870	HM536765
Cyclocheilichthys enoplos	JX066753	JX066771	JX066784	JX066797	JX066737
Puntioplites falcifer	HM536904	HM536805	HM536726	HM536868	HM536762
Puntioplites proctozystron	HM536912	HM536813	HM536732	HM536879	HM536770
Puntioplites waandersi	HM536928	HM536829	HM536747	HM536863	HM536786
“Group B”					
Acrossocheilus cinctus	JX066754	JX066772	JX066785	JX066798	JX066738
Acrossocheilus monticola	HM536893	HM536795	HM536715	HM536839	HM536756
Barbonymus altus	JX066755	JX066773	JX066786	JX066799	JX066739
Barbonymus gonionotus	AB238966	AB238966	AB238966	AB238966	AB238966
Barbonymus schwanenfeldii	HM536894	HM536796	HM536716	HM536875	HM536757
Poropuntius bantamensis	JX066756	JX066774	JX066787	JX066800	JX066740
Poropuntius normani	JX066757	JX066775	JX066788	JX066801	JX066741
Poropuntius opisthopterus	HM536891	HM536793	HM536713	HM536837	HM536755
Hypsibarbus malcolmi	HM536915	HM536816	HM536735	HM536872	HM536773
Hypsibarbus vernayi	HM536892	HM536794	HM536714	HM536838	DQ845870
Hypsibarbus wetmorei	JX066758	JX097078	JX097086	JX097086	N/A
Other taxa					
Barbus barbus	AB238965	AB238965	AB238965	AB238965	AB238965
Barbus carnaticus	HM010713	HM010725	HM010737	HM010737	HM010701
Barbus fasciolatus	HM536910	HM536811	HM536730	HM536877	HM536768
Barbus trimaculatus	AB239600	AB239600	AB239600	AB239600	AB239600
Capoeta capoeta	HM536852	HM536882	HM536707	HM536850	HM536751
Catlocarpio siamensis	HM536911	HM536812	HM536731	HM536878	HM536769
Eirmotus octozona	HM536918	HM536819	HM536737	HM536855	HM536776
Hampala macrolepidota	HM536886	HM536790	HM536709	HM536832	DQ845863
Labeobarbus caudovittatus	JX066759	JX097076	JX097087	JX097087	JX066742
Labeobarbus intermedius	JX066760	JX097074	JX097085	JX097085	JX066743

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TABLE 1. (Continued)

Taxa	COI	Cyt *b*	ND4	ND5	16S
Mystacoleucus marginatus	HM536913	HM536814	HM536733	HM536880	HM536771
Neolissochilus hexagonolepis	JX066761	JX097077	JX097088	JX097088	JX066744
Neolissochilus stracheyi	HM536922	HM536823	HM536741	HM536857	HM536780
Onychostoma simum	HM536899	HM536801	HM536721	HM536843	DQ845861
Oreichthys cosuatis	HM536921	HM536822	HM536740	HM536856	HM536779
Probarbus jullieni	HM536909	HM536810	HM536729	HM536848	HM536767
Puntius brevis	HM536914	HM536815	HM536734	HM536871	HM536772
Puntius nigrofasciatus	HM536920	HM536821	HM536739	HM536849	HM536778
Puntius oligolepis	HM536919	HM536820	HM536738	HM536881	HM536777
Puntius tetrazona	EU287909	EU287909	EU287909	EU287909	EU287909
Puntius ticto	AB238969	AB238969	AB238969	AB238969	AB238969
Puntius titteya	HM536908	HM536809	HM536728	HM536876	HM536766
Scaphognathops bandanensis	HM536927	HM536828	HM536746	HM536862	HM536785
Scaphognathops stejnegeri	HM536906	HM536807	HM536705	HM536847	HM536764
Sikukia stejnegeri	HM536898	HM536800	HM536720	HM536842	DQ845872
Sinocyclocheilus altishoulderus	FJ984568	FJ984568	FJ984568	FJ984568	FJ984568
Sinocyclocheilus grahami	GQ148557	GQ148557	GQ148557	GQ148557	GQ148557
Sinocyclocheilus macrophthalmus	HM536889	HM536792	HM536711	HM536835	HM536754
Sinocyclocheilus microphthalmus	HM536888	AY854690	HM536703	HM536834	HM536753
Sinocyclocheilus xunlensis	HM536887	HM536791	HM536710	HM536833	HM536752
Spinibarbus hollandi	HM536890	AY195629	HM536712	HM536836	DQ845865
Spinibarbus sinensis	HM536895	HM536797	HM536717	HM536840	DQ845864
Tor sinensis	HM536900	HM536802	HM536722	HM536844	DQ845876
Tor tambroides	HM536923	HM536824	HM536742	HM536858	HM536781
Varicorhinus mariae	JX066762	JX066776	JX066789	JX066802	JX066745
Varicorhinus sandersi	JX066763	JX097075	JX097089	JX097089	N/A
Tribe Cyprinini					
Carassioides acuminatus	AP011178	AP011178	AP011178	AP011178	AP011178
Carassius auratus	AB111951	AB111951	AB111951	AB111951	AB111951
Carassius carassius	AY714387	AY714387	AY714387	AY714387	AY714387
Carassius cuvieri	AB045144	AB045144	AB045144	AB045144	AB045144
Carassius gibelio	AB379922	AB379922	HM536748	AB378300	AB379922
Carassius langsdorfii	AB006953	AB006953	AB006953	AB006953	AB006953
Cyprinus carpio	X61010	X61010	X61010	X61010	X61010
Cyprinus multitaeniatus	HM536896	HM536798	HM536718	HM536866	DQ845845
Procypris rabaudi	EU082030	EU082030	EU082030	EU082030	EU082030
Tribe Oreinini					
Chuanchia labiosa	HM536897	HM536799	HM536719	HM536841	HM536758

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TABLE 1. (Continued)

Taxa	COI	Cyt *b*	ND4	ND5	16S
Gymnocypris przewalskii	AB239595	AB239595	AB239595	AB239595	AB239595
Oxygymnocypris stewartii	HM536853	DQ491114	HM536749	HM536864	DQ845918
Platypharodon extremus	HM536854	AY463498	HM536750	HM536851	DQ845855
Schizothorax oconnori	HM536902	AY463519	HM536724	HM536846	HM536760
Schizothorax waltoni	HM536903	HM536804	HM536725	HM536867	HM536761
Tribe Labeonini					
Cirrhinus microlepis	HM536924	HM536825	HM536743	HM536859	HM536782
Crossocheilus reticulatus	HM536925	HM536826	HM536744	HM536860	HM536783
Epalzeorhynchos bicolor	HM536917	HM536818	HM536706	HM536874	HM536775
Garra orientalis	AP011202	AP011202	AP011202	AP011202	AP011202
Lobocheilos melanotaenia	HM536901	HM536803	HM536723	HM536845	HM536759
Labeo batesii	AB238967	AB238967	AB238967	AB238967	AB238967
Labeo senegalensis	AB238968	AB238968	AB238968	AB238968	AB238968
Labiobarbus lineatus	HM536885	HM536789	HM536708	HM536865	DQ845914
Osteochilus salsburyi	HM536883	HM536787	HM536701	HM536830	DQ845892

Sequence alignment, phylogenetic analyses and hypotheses testing. Multiple alignment of the protein-coding gene sequences was performed with the help of CLUSTAL X (Thompson *et al.* 1997) and SEAVIEW alignment editor (Galtier *et al.* 1996). Alignment of the 16S rRNA gene followed the methods of Li *et al.* (2008). Partitioned ML search was performed using RAxML v.7.0.4 (parallelized version) (Stamatakis 2006) on the high performance cluster computing facility (36 nodes) located at Saint Louis University. We used a mixed model analysis; nucleotide data were partitioned into 14 partitions according to codon positions of each of the four protein-coding genes and the stems and loops of 16s rRNA gene. The GTR+Γ+I model (with 4 discrete rate categories) was chosen for each partition. A total of 100 distinct runs were performed based on 100 random starting trees using the default settings of the program. The tree with the highest maximum likelihood score was selected as the final tree. ML bootstrap analysis was conducted using RAxML (Felsenstein 1985; Stamatakis *et al.* 2008). Rapid bootstrapping algorithm and GTRCAT approximation were used in the analyses. The same partitioning strategy was used as in the initial maximum likelihood search. The number of nonparametric bootstrap replications was set at 1,000, and other parameters were set as default. The resulting trees were imported into PAUP*4.0.b10 (Swofford 2002) to obtain the 50% majority rule consensus tree.

A constraint tree was constructed according to the following five hypotheses summarized from Rainboth (1989): 1) each of the three genera *Discherodontus*, *Chagunius*, and *Hypselobarbus* is monophyletic; 2) *Chagunius* and *Discherodontus* are sister clades; 3) the three genera form a monophyletic group; 4) the clade formed by the three genera and the genera included in “Group A” form a clade; and 5) taxa in “Group B” constitute a monophyletic group. Partitioned ML searches were then carried out using RAxML. Data were partitioned into 14 sections as before and the GTR+Γ+I model was adopted for each partition. A total of 100 distinct runs were performed based on constraint tree. The tree with the greatest maximum likelihood score was chosen as the best tree. The site-wise log-likelihood scores were calculated for the best ML tree resulting from the original unconstraint search and the best ML tree obtained from the constrained search using PAUP and used as input for CONSEL v.0.20 (Shimodaira & Hasegawa 2001). An approximately unbiased (AU) test was then conducted using CONSEL to investigate whether the latter tree significantly worse than the former.

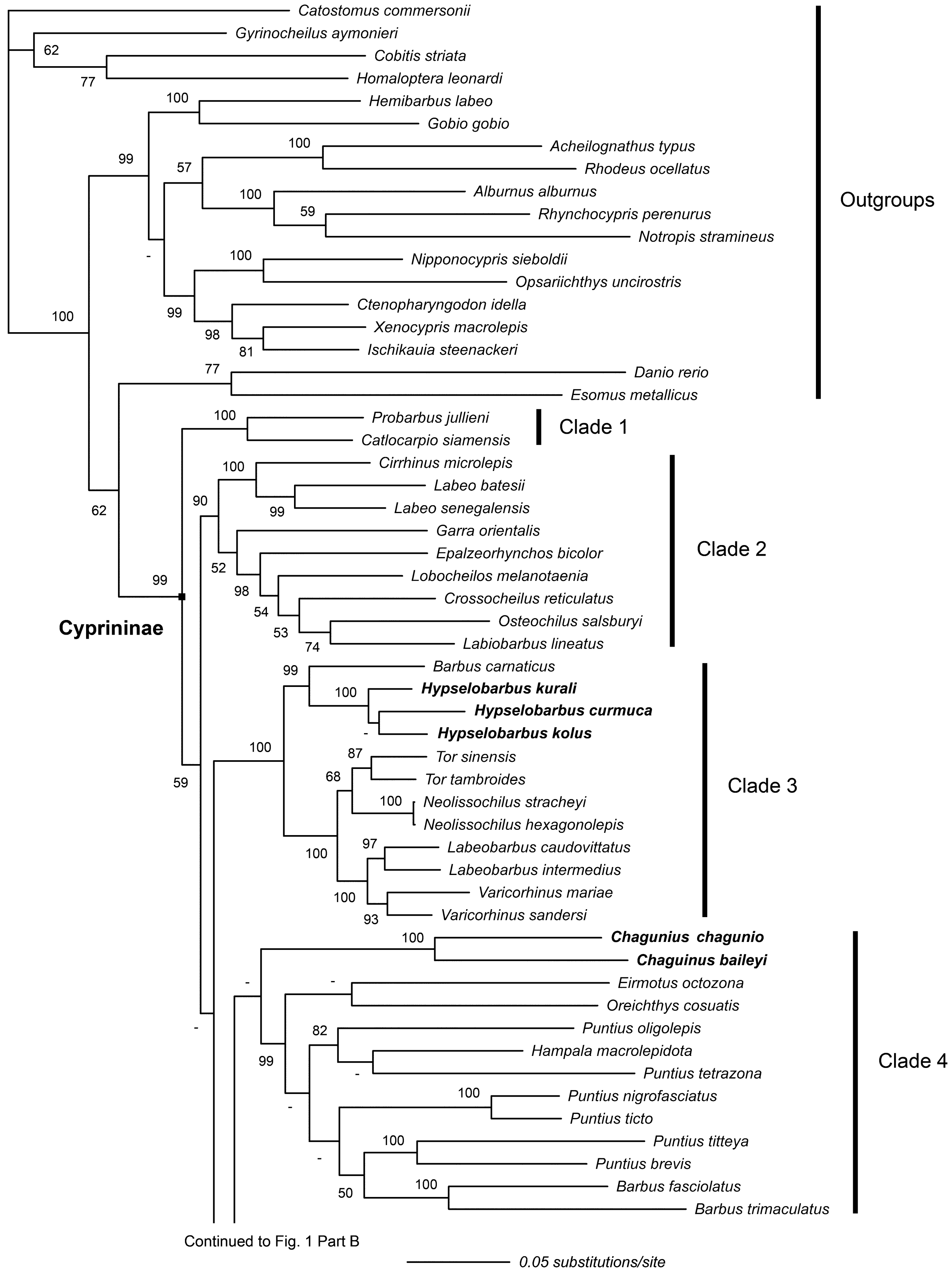
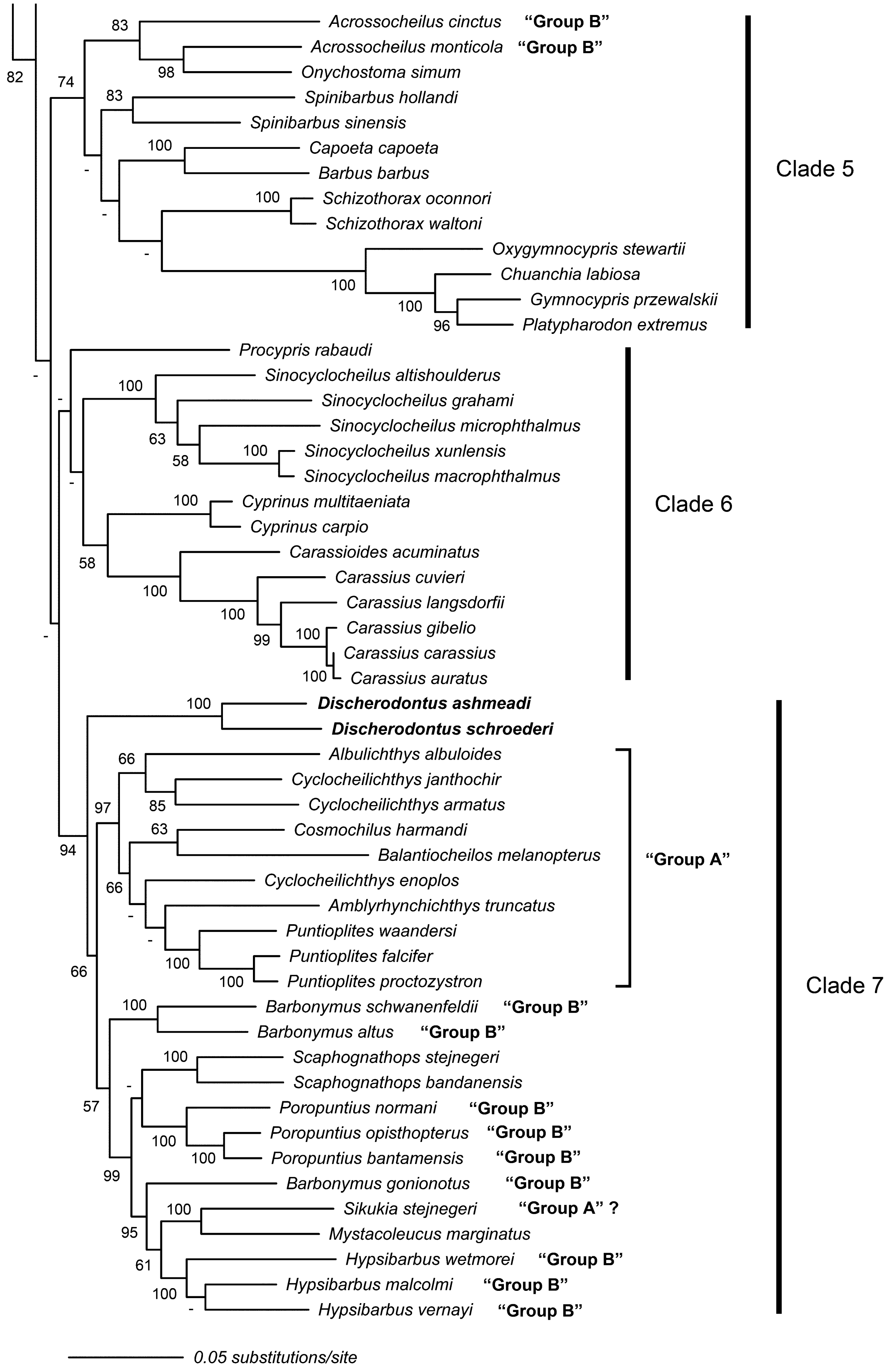


FIGURE 1. The best likelihood tree (-ln *L* = 154361.268266) resulting from partitioned maximum likelihood analysis. Numbers at branches indicate the bootstrap values from the maximum likelihood bootstrap analysis (1,000 replicates). "–" denotes node with support value lower than 50%. Species of the genera *Discherodontus, Chagunius,* and *Hypselobarbus* are highlighted in bold. Taxa included in "Group A" and "Group B" (*sensu* Rainboth 1989) are indicated on the tree.

Continued from Fig. 1 Part A



Results

A total of 5601 bp nucleotides were sequenced for the five mitochondrial genes: COI (678 bp), Cyt *b* (1141 bp), ND4 (1381 bp), ND5 (1842 bp), and 16S (559 bp/nt). For 16S rRNA, 302 bp belong to stems, and 257 nt belong to loops. Nucleotide sequences of the first four protein-coding genes code for 226, 380, 460, and 614 amino acids, respectively. For the whole dataset, 3239 characters were variable, and 2832 characters were parsimony informative. Mean base frequencies of the whole dataset were A, 0.30495; C, 0.27304; G, 0.15214, and T, 0.26987. The optimal maximum likelihood tree has a likelihood value of -ln *L* = 154361.268266.

The subfamily Cyprininae was resolved as monophyletic with a bootstrap value (BP) of 99%. Seven major clades (1–7) were recovered in this subfamily (Fig. 1). The two species of *Discherodontus* were sister to each other (BP = 100%) and together formed the base of the strongly supported clade 7 (BP = 96%). The two species of *Chagunius* formed a monophyletic group (BP = 100%) basal to clade 4, a clade that was only weakly supported (BP < 50%). All three species of *Hypselobarbus* formed a strongly supported clade (BP = 100%) in the robustly supported (BP = 100%) clade 3 and was sister to *Barbus carnaticus* (BP = 100%), and they together formed a clade sister to the clade composed of *Tor*, *Neolissochilus*, *Labeobarbus*, and *Variorhinus* (Fig. 1). All “Group A” taxa, i.e. *Albulichthys*, *Amblyrhynchichthys*, *Balantiocheilos*, *Cosmochilus*, *Cyclocheilichthys* and *Puntioplites*, formed a strongly supported (BP = 99%) subclade in clade 7. The genus *Puntioplites* is monophyletic (BP = 100%), but the genus *Cyclocheilichthys* is not monophyletic. *Albulichthys* is monotypic. The remaining three genera only have one representative each in this analysis. *Sikukia* was also in clade 7 but not in the subclade formed by “Group A” taxa. For the “Group B” taxa, *Acrossocheilus* is in clade 5 (BP = 88%), whereas *Barbonymus*, *Poropuntius* and *Hypsibarbus* are allied in clade 7. The two *Acrossocheilus* species, *A. cinctus* and *A. monticola*, did not form a clade. *Barbonymus altus* and *B. schwanenfeldii* are sister species (BP = 100%), but *B. gonionotus* did not group with them. The three species of *Poropuntius* formed a monophyletic group (BP = 100%), as did the three species of *Hypsibarbus* (BP = 100%). The optimal maximum likelihood tree resulting from the constraint search has a likelihood value of -ln *L* = 155357.832450. The AU test showed that this tree is significantly worse than the optimal ML tree (-ln *L* = 154361.268266) obtained from the original unconstraint search (-ln *L* difference = 996.564184; *p* = 0.00004).

Discussion

Placements and phylogenetic relationships. Molecular phylogenetic analysis of the taxa sampled in this study provides insight into the sister group relationships of this relatively unknown group of Cypriniformes and significantly aids in clarification of taxonomic and classification issues that have long been unresolved. Our conclusions are tempered by the fact that the phylogenetic hypothesis supported by our analysis is based solely on mitochondrial genes and thus represents data from what is essentially a single locus. It is well established that phylogenetic analyses based on a single locus may result in a gene tree that differs from the true species tree due to the stochasticity of the coalescent process (Funk & Omland 2003). In addition, the rapid evolution of mtDNA, while providing resolution to shallow branches of a phylogenetic tree, may result in poorly resolved deeper nodes due to saturation. As described above, the use of nuclear loci were precluded by the polyploidy observed in many cyprinine taxa making assessment of homology difficult. Future research on this group should include the development of nuclear loci and resolution of issues associated with polyploidy to construct a multi-locus species tree that may better represent the genealogical relationships of the taxa in this clade (Knowles 2009; Edwards 2009).

The monophyly of the individual genera *Discherodontus*, *Hypselobarbus*, and *Chagunius*, corroborates the hypothesis of Rainboth (1989). However, the three monophyletic genera were not grouped together and the first two genera are not sister to each other. These three genera were actually distributed in three different clades (clade 7, clade 4, and clade 3 respectively) of the phylogenetic tree and clade 3 and clade 7 are strongly supported (Fig. 1). These results contradict the hypotheses proposed by Rainboth (1989), which stated that *Discherodontus* is most closely related to *Chagunius*, the next closely related taxon is *Hypselobarbus*, and these three genera form a monophyletic group.

Because our results show that *Discherodontus*, *Chagunius*, and *Hypselobarbus* are distantly related to one another, it is inappropriate to treat them as a group and evaluate the relationships between them and those genera included in “Group A” (*Albulichthys*, *Amblyrhynchichthys*, *Balantiocheilus*, *Cosmochilus*, *Cyclocheilichthys*, *Neobarynotus,* and *Puntioplites*) and “Group B” (*Acrossocheilus, Barbonymus, Poropuntius,* and *Hypsibarbus*). In our phylogenetic tree, all genera in “Group A” (except the unsampled *Neobarynotus*) form a monophyletic group in clade 7. Rainboth (1989), however, did not claim that “Group A” was monophyletic, because none of the 12 morphological characters he examined were shared only by taxa of this group. Rainboth (1989) suggested *Sikukia* as a possible member of “Group A.” However, in our phylogenetic tree, this genus was not part of the subclade formed exclusively by “Group A” taxa. Finally, “Group B” is not monophyletic, a result contradictory with the opinion of Rainboth (1989).

Rainboth (1981) placed *Protopuntius* (= *Discherodontus*) and *Chagunius* in his tribe Barbini subtribe Barbi. In our phylogenetic tree, most genera in clade 7 and some genera in clade 5 (e.g. *Barbus*, *Capoeta*), clade 6 (e.g. *Sinocyclocheilus*), and clade 1 (*Probarbus*) were included in his subtribe Barbi. All members of “Group A” and “Group B” *sensu* Rainboth (1989) can be found in this subtribe (*Neobarynotus* was not examined herein and *Hypsibarbus* was not described until 1996). Rainboth (1981) classified *Gonoproktopterus* (= *Hypselobarbus*) in his tribe Babini subtribe Tores. The genera *Tor* and *Mesopotamichthys* were also listed within this subtribe with *Varicorhinus* and *Carasobarbus* as two other possible members. Three genera, *Hypselobarbus*, *Tor* and *Varicorhinus*, were sampled in our current study and they all located in clade 3.

Rainboth (1991) provided a classification for Southeast Asian cyprinids. The three genera *Discherodontus*, *Chagunius*, and *Hypselobarbus* were placed in his tribe Systomini and subtribe Poropuntii. All four genera included in “Group B” were also put in the subtribe Poropuntii. All genera (except *Neobarynotus*) in “Group A” were in the tribe Systomini and subtribe Osteobramae. Possibly, the genus *Neobarynotus* was treated as a synonym of *Cyclocheilichthys* by Rainboth following Roberts (1989). Rainboth (1991) proposed a close relationship between *Discherodontus*, *Chagunius*, and *Hypselobarbus* and taxa in “Group B,” because they were placed in the same subtribe. This opinion is different from that of his earlier study (Rainboth, 1989), where he suggested that these three genera were distantly related to “Group B” taxa but were closely related to “Group A” taxa.

Rainboth (1996) studied the classification of fishes of the Cambodian Mekong. No species of *Hypselobarbus* or *Chagunius* occur in that drainage. The genus *Discherodontus*, all members of “Group A” (except *Neobarynotus*), *Sikukia* and the genus *Mystacoleucus* constitute the entire subtribe Osteobramae (tribe Systomini). Three genera of “Group B,” i.e. *Barbodes* (*Barbonymus*), *Poropuntius*, and *Hypsibarbus* were included in the study and were placed in his tribe Systomini subtribe Semiploti. Results from the present molecular study do not support any of the hypotheses of Rainboth (1981), Rainboth (1989), Rainboth (1991), or Rainboth (1996). As can be seen from our molecular phylogeny, *Discherodontus* is actually closely related to all genera (except *Acrossocheilus*) in both “Group A” and “Group B” because they are all located in clade 7, whereas *Hypselobarbus* and *Chagunius* are only distantly related to these genera. The phylogenetic position of *Chagunius* may change with increased taxon and genomic sampling, because its placement in clade 4 is only weakly supported.

It is noteworthy that, historically, there has been a debate on the nomenclatorial status of the name *Hypselobarbus*. Rainboth (1989) discussed this in some detail. The debate focuses on whether the type species of *Hypselobarbus*, *Barbus mussullah*, belongs to the genus *Tor* (Hora 1942, 1943) or not (Bleeker 1860; Rainboth 1989). If *B. mussullah* is found to be a member of *Tor*, then *Hypselobarbus* becomes a junior synonym of *Tor*; if *B. mussullah* is grouped with other species of *Hypselobarbus*, then *Hypselobarbus* is a valid genus. As can be seen from our phylogenetic tree, all sampled species of *Tor* and *Hypselobarbus* were located in clade 3. They are only distantly related to *Discherodontus*, *Chagunius* and genera included in “Group A” and “Group B.” That is to say, the nomenclatorial status of *Hypselobarbus* will not alter our conclusions regarding the phylogenetic relationships between members of this genus and members of *Discherodontus*, *Chagunius* and genera contained in those two taxonomic groups.

Distribution and homoplasy of morphological characters. Rainboth (1989) stated that all barbin genera have three rows of pharyngeal teeth, except *Discherodontus* that has two rows and *Probarbus* that has one row. He thought that the possession of two rows of pharyngeal teeth could indicate monophyly of the genus *Discherodontus*. He also suspected that *Discherodontus* and *Probarbus* were not closely related, and used homoplasy to explain why *Probarbus* also had fewer than three rows of pharyngeal teeth. After thorough literature review, we found that there are actually more barbin genera that may have fewer than three rows of pharyngeal teeth, including: *Barbopsis* (2 rows; Banister & Bunni 1980), *Caecobarbus* (2 rows; Thinès 1969), *Pseudobarbus*

(2 or 3 rows; Skelton 2001), *Scaphognathops* (2 rows; Smith 1945), *Typhlobarbus* (2 rows; Shan *et al.* 2000), *Aulopyge* (1 row; Ćaleta *et al.* 2009), *Catlocarpio* (1 row; Smith 1945) and *Sawbwa* (1 row; Talwar & Jhingran 1991). According to our phylogenetic tree, barbin fishes do not form a monophyletic group as they can be found in almost all major clades except clade 2 which is exclusively comprised of labeonins. Therefore, it is necessary to know whether other cyprinine fishes traditionally not considered as barbins also possess fewer than three rows of pharyngeal teeth. Following further literature review, we identified the following genera with fewer than three rows of teeth: *Carassius* (1 row; Luo & Yue 2000), *Carassioides* (2 rows; Luo & Yue 2000), *Discocheilus* (2 rows; Zhang *et al.* 2000), *Discogobio* (2 rows; Zhang *et al.* 2000), *Hongshuia* (2 rows; Zhang *et al.* 2008), *Horalabiosa* (2 rows; Silas 1954), *Mekongina* (2 rows; Fowler 1937), *Paracrossocheilus* (2 rows; Weber & de Beaufort 1916), *Parapsilorhynchus* (2 rows; Talwar & Jhingran 1991), *Phreatichthys* (2 rows; Vinciguerra 1924), *Placocheilus* (2 rows; Zhang *et al.* 2000), *Ptychidio* (2 rows; Myers 1930), *Sinocrossocheilus* (2 rows; Zhang *et al.* 2000), *Typhlogarra* (2 rows; Banister & Bunni 1980), *Chuanchia* (2 rows; Chen & Cao 2000), *Diptychus* (2 rows; Chen & Cao 2000), *Gymnocypris* (2 rows; Chen & Cao 2000), *Gymnodiptychus* (2 rows; Chen & Cao 2000), *Oxygymnocypris* (2 rows; Chen & Cao 2000), *Platypharodon* (2 rows; Chen & Cao 2000), *Ptychobarbus* (2 rows; Chen & Cao 2000), *Schizopygopsis* (2 rows; Chen & Cao 2000), and *Herzensteinia* (1 row; Chen & Cao 2000). Among all the genera listed above, nine genera *Probarbus*, *Catlocarpio*, *Scaphognathops*, *Carassius*, *Carassioides*, *Chuanchia*, *Gymnocypris*, *Oxygymnocypris* and *Platypharodon* were sampled in the present study. The genera *Catlocarpio* and *Probarbus*, both with one pharyngeal tooth row, form clade 1; *Carassius* and *Carassioides* were found in clade 6; *Chuanchia*, *Gymnocypris*, *Oxygymnocypris* and *Platypharodon* occur in clade 5. The genus *Scaphognathops* possesses the same number of rows of pharyngeal teeth as *Discherodontus* and is also located in clade 7. It should also be noted that, although the number of rows of pharyngeal teeth is usually the same in a single species, it can vary within a genus. For example, in the genera *Pseudobarbus* and *Garra*, there may be 2 or 3 rows of pharyngeal teeth (Skelton 2001, Zhou *et al.* 2005). The genera *Cyprinus* and *Schizothorax* both have species with 3 or 4 rows of pharyngeal teeth (Luo & Yue 2000; Chen & Cao 2000). As a summary, the possession of two rows of pharyngeal teeth alone cannot be used to support the monophyly of *Discherodontus*; however, our molecular data support the monophyly of this genus given current taxon sampling.

Rainboth (1989) also argued that the possession of two characters, 5 unbranched dorsal-fin rays and 4 unbranched anal-fin rays, could support the monophyly of the genus *Chagunius*. This hypothesis was drawn mainly from his observation that the number of unbranched dorsal and anal-fin rays in *Discherodontus*, *Hypselobarbus*, and all genera of "Group A" and "Group B" is 4 and 3, respectively. We performed an extensive literature review of the number of unbranched dorsal and anal-fin rays for most cyprinine species and also examined available specimens of cyprinine taxa. The possession of 5 unbranched dorsal-fin rays seems unique to *Chagunius*. As to the number of unbranched anal-fin rays, according to Talwar & Jhingran (1991), it is 4 in *Chagunius baileyi*, but it is usually 3 in *C. chagunio* and *C. nicholsi*. Therefore, possession of 5 unbranched dorsal-fin rays can be used as a character to diagnose *Chagunius*.

Rainboth (1989) claimed that the monophyly of *Hypselobarbus* is indicated by one character, the possession of 9 branched dorsal-fin rays. His conclusion was mainly drawn from his observation that the number of branched dorsal-fin rays in *Discherodontus*, *Chagunius*, and all genera of the "Group A" and "Group B" is 8. We did an extensive search of the literature for the number of branched dorsal-fin rays in cyprinine fishes and also examined available specimens. It is confirmed that all species of *Hypselobarbus* have 9 branched dorsal-fin rays, and all species of *Discherodontus* and *Chagunius* only have 8. However, all genera (except *Albulichthys* and *Neobarynotus*) in "Group A" have one or more species that may possess 9 branched dorsal-fin rays. We use the word 'may' because the number of branched dorsal-fin rays sometimes varies between 8 and 10 even in a single species. For "Group B," all species of the four genera included have 8 branched dorsal-fin rays except the species *Barbus* (*Barbodes*) *bovanicus* that possess 8–10 branched dorsal-fin rays (Talwar & Jhingran 1991; Menon 1999). We also noticed that most species of *Tor*, *Neolissochilus*, and *Spinibarbus* have 9 branched dorsal-fin rays. Some other barbin genera (e.g. *Sinocyclocheilus*, *Paraspinibarbus*) have members with 9 branched dorsal-fin rays. Some labeonin genera (e.g. *Gibelion*, *Garra*) and some oreinin genera (e.g. *Schizothorax*, *Ptychobarbus*, *Schizopygopsis*) also have members with this character. In summary, the possession of 9 branched dorsal-fin rays is not a character unique to the genus *Hypselobarbus*. Rather, this character is commonly found in cyprinine fishes; thus it cannot be used to support the monophyly of *Hypselobarbus*.

Rainboth (1989) argued that the monophyly of the group formed by *Discherodontus*, *Chagunius*, and *Hypselobarbus* is supported by the possession of 1) prone gill rakers and 2) a black apex on the dorsal fin. After examining the first character in some barbins, we found this character is quite variable and sometimes it is hard to determine whether the gill rakers are prone or erect. For the second character, Rainboth (1989) found that some species of *Barbodes* also have a black apex on the dorsal fin. As an explanation, he argued that: "The presence of similar coloring in some species of *Barbodes* (Group B) is presumably a homoplasy, since monophyly of group B is supported by character 9, modified jaw morphylogy" (Rainboth 1989: 28). According to our phylogeny, it is true that the genus *Barbodes* (*Barbonymus*) did not group with *Discherodontus*, *Chagunius*, or *Hypselobarbus*, and the presence of a black apex on the dorsal fin of some species of *Barbodes* is better explained through the homoplasy hypothesis. However, we cannot agree with Rainboth's explanation, because "Group B" itself is not monophyletic. Our molecular phylogeny does not support the hypothesis that *Discherodontus*, *Chagunius*, and *Hypselobarbus* form a monophyletic group. Therefore, the combination of the two characters that Rainboth (1989) used (i.e. possession of prone gill rakers and possession of a black apex on the dorsal fin) might have evolved independently multiple times in cyprinines.

Rainboth (1989: 28) stated that: "Relationship between group A, *Discherodontus*, *Hypselobarbus*, and *Chagunius* is suggested by the long bases of the gill rakers …" However, our phylogenetic analysis reveals that these taxa do not form a monophyletic group (Fig. 1). The long bases of the gill rakers found in these taxa would be better explained by a hypothesis of convergence or parallelism. Rainboth (1989) also proposed that one character, modified jaw morphology (i.e. demarcation between lower lip and jaw), could be used to support the monophyly of "Group B." The homology of this proposed character will need further evaluation with greater taxon sampling, as our results from the molecular phylogenetic analyses do not corroborate the hypothesis that taxa in "Group B" constitute a monophyletic group. Therefore, at this point in time we hypothesize that this character evolved independently multiple times; the functionality of this modified jaw morphology will likely aid in determining if it is homologous across these taxa and thus in conflicting with the molecular data or if it is convergent and has evolved independently across different lineages.

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