Phylogenetic studies of Chinese labeonine fishes (Teleostei: Cyprinidae) based on the mitochondrial 16S rRNA gene*

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Abstract The mitochondrial 16S ribosomal RNA gene is sequenced from 24 ingroups taxa, including 18 species from Labeoninae grouped in 13 genera. Phylogenetic analyses are subjected to neighbor joining, maximum parsimony, maximum likelihood and Bayesian analyses. Phylogenetic analysis indicates that Labeoninae is basically a monophyletic assemblage and can be divided into 2 major clades: one comprising the genera *Cirrhinus*, *Crossocheilus* and *Garra*; and the other consisting of the genera *Labeo*, *Sinilabeo*, *Osteochilus*, *Pseudorossocheilus*, *Parasinilabeo*, *Ptychidio*, *Semilabeo*, *Pseudogyricheilus*, *Rectori* and *Discogobio*. According to our present analysis, the features such as the presence of the adhesive disc on the chin and the pharyngeal teeth in 2 rows used in the traditional taxonomy of Labeoninae provide scarce information for phylogeny of labeonine fishes.

Keywords: 16S rRNA, Labeoninae, monophyly, phylogeny, Cyprinidae.

Labeoninae, one of the subfamilies contained within Cyprinidae, is a huge group including more than 300 species in approximately 26 genera. Labeonine fishes are highly adaptive to torrential habitats, and they are mainly distributed in the tropical and subtropical regions of Africa and Asia[1]. The labeonine known in Africa is a diversified group and comprises about 120 species and subspecies placed in 2 genera, *Labeo* and *Garra*[2]. This assemblage includes 20 genera over 60 species in China, within which there are 8 genera endemic to China[1]. In China, labeonine fishes are mainly distributed in the water systems in south of the Yangtze River. The subfamilies Labeoninae and Barbininae, together with most of the genera of Danioninae and the genus *Puntioplites* of Cyprininae, constitute the south Asian group[1].

Labeonins had a taxonomic difficulty because of the lack of appropriate taxonomic features for assignation of these included genera to higher taxonomic categories. Labeonine fishes were affiliated to the subfamily Barbininae[3]. Based on morphology of barbels and the pattern of innervations correlated with barbels, Howes placed labeonins into the subfamily Cyprininae[4]. On account of the peculiar morphology of lips and associated structures adapted for torrential inhabit and the pharyngeal teeth in 2 rows, this assemblage was recognized as Garrinae. These two characters are, however, irregularly distributed among the species of labeonine fishes, so they cannot be used as diagnostic for species otherwise than garrini group[3].

Based on osteological synapomorphy, Chen et al. treated Labeoninae as a monophylotic assemblage within Cyprinidae, and placed Labeoninae in Barbini Series[5]. Because of the increase of the new records of species and its important role in economy, Labeonins has received considerable taxonomical attention[6—8]. However, all the previous phylogenies were focused on a fraction of labeonine species only. Therefore, up to date no subsequent work has been done to provide phylogenetic information on the taxa of this group.

The mitochondrial DNA has been widely used in the studies of molecular systematics of vertebrates[9,10], and the mitochondrial 16S rRNA gene sequence was used as an effective genetic marker[11,12]. In this study, we used the sequences of the mitochondrial DNA 16S rRNA for 18 labeonine species to test the monophyly of Labeoninae, and we also aimed to construct phylogenetic relationships of labeonine fishes at the genus level.

1 Materials and methods

1.1 Sample collection

A total of 27 species, including 18 species in 13 genera from Labeoninae and the others from the sum-

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family Schizothoracinae, Barbininae and Cyprininae, were used for this study. The data of *Cyprinus carpio* (NC001606) and *Carassius auratus* (NC002079) were retrieved from GenBank. *Myxocyprinus asiaticus* was chosen as the outgroup owing to its clear relationship with family Cyprinidae. Locations of origin and other relevant information are given in Table 1. All the tissues used for extraction of DNA were preserved in 95% ethanol and most of them were deposited in the Freshwater Fish Museum of Institute of Hydrobiology, Chinese Academy of Sciences.

Table 1. Species identifications, locations of origin and the length of analyzed sequence

Taxons	Species	Location	Length (bp)
Labeoninae	*Cirrhinus molitorella*	Tengxian, Guangxi	1208
	Osteochilus salsburyi	Rongan, Guangxi	1211
	Sinilabeo laticeps	Mengla, Yunnan	1176
	Crossocheilus latius	Tengchong, Yunnan	1215
	Labeo yunnanensis	Mengla, Yunnan	1217
	Pseudorossocheilus bamaensis	Tiane, Guangxi	1243
	Parasinilabeo assinilabeo	Rongan, Guangxi	1182
	Ptychidio jordani	Tiane, Guangxi	1144
	Semilabeo notabilis	Tiane, Guangxi	1121
	Pseudogyrincheilus procheilus	Luding, Sichuan	1274
	Rectoris posehensis	Duan, Guangxi	1258
	Discogobio laticeps	Tiane, Guangxi	1149
	Discogobio bismargaritus	Tiane, Guangxi	1157
	Discogobio brachyphysallidos	Jinxiu, Guangxi	1148
	Discogobio tetrabarbatus	Rongan, Guangxi	1271
	Garra pingi hainanensis	Changhua, Ledong	1174
	Garra kempi	Chayu V, Tibet	1149
	Garra orientails	Changhua, Ledong	1229
Barbininae	*Onychostoma sima*	Hejiang, Sichuan	1254
	Spinibarbus hollandi	Tunxi, Huangshan	1148
Cyprininae	*Procypris rabaudi*	Hejiang, Sichuan	1215
	Carassius auratus	NC 002079	1839
	Cyprinus carpio	NC 001606	1839
	Cyprinus multitaeniata	Guiping, Guangxi	1234
Schizothoracinae	*Schizothorax waltoni*	Chayu Ⅳ, Tibet	1341
	Schizothorax molesworthi	Chayu V, Tibet	1247
Family Catostomidae	*Myxocyprinus asiaticus*	Wuhan, Hubei	1193

1.2 DNA extraction, PCR amplification and sequencing

Total DNA was extracted from muscle tissues or fins following Ausubel's method[13]. The following primers, 16SL (5′-CTT ACA CCG AGA ARA CAT C-3′) and 16SH (5′-CTT AAG CTC CAA AGG GTC-3′), were used for amplification and sequencing. Amplification reactions were performed in a 60μL volume containing approximately 100 ng of template DNA, 0.75 μL dNTP (2.5 mmol/L of each), 1.5μL of each primer, 5μL of 10 X reaction buffer and 3 units of *Taq* DNA polymerase (Biostar). The thermocycling conditions included an initial denaturation at 95 ℃ for 3 min, followed by 30 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min and were completed with a final extension at 72 ℃ for 5 min. The amplified fragments were fractionated by electrophoresis through 0.8% low-melting agarose gels, recovered from the gels and purified using Biostar Glassmilk DNA purification Kit following the manufacture's instructions. The purified fragments were directly sequenced in both forward and reverse directions by Shanghai Biostar Genechip Inc.

1.3 Sequence analysis

Multiple alignments of sequences were performed using Clustal W[14] with a gap-opening penalty of 20 and a gap-extension penalty of 20 and Seaview[15] alignment editor and verified by eye. Measures of nucleotide composition were carried out by MEGA2.1 software[16]. The mutational saturation for nucleotide substitutions was examined by plotting transversions and transitions against Kimura two-parameter distance using DAMBE4.1[17]; saturation was estimated by the extent to which the slope of a linear regression departs from a value of 1. Aligned sequences were analyzed using maximum parsimony (MP), neighbor

joining (NJ), maximum likelihood (ML) and Bayesian methods. All phylogenetic analyses including maximum parsimony, neighbor joining and maximum likelihood were performed with PAUP 4.0b10[18] except for Bayesian method, the latter was carried out with MrBayes3.0b4[19].

Maximum parsimony search was conducted using heuristic search methods with tree bisection-reconnection (TBR) branch swapping, and 10 random sequence addition replicates. Non-parametric bootstrap analyses with 1000 pseudoreplicates and 10 random sequence additions were conducted on the equally weighted data. In order to determine which model is the best-fit, we selected models using Modeltest 3.06[20]. Finally, the GTR model which incorporates rate variation (Γ) along with the number of invariable sites (I) was utilized for neighbor joining and maximum likelihood analyses based on a hierarchical hypothesis test of alternative models implemented with modeltest 3.06. The *Ti/ Tv* ratio, gamma shape parameter and proportion of non-variant sites were estimated by maximum likelihood from a maximum parsimony tree. After the best-fit model was found, we performed a heuristic search using the same branch swapping as described previously for NJ and ML. In ML analysis, starting tree was obtained via neighbor joining, then the bootstrap tests were performed again, 1000 for NJ analysis and 100 for ML analysis.

Bayesian analysis using Markov Chain Monte Carlo (MCMC) was performed using MrBayes 3.0b4. To decrease the chance of reaching apparent stationary on local optima, two separate analyses were conducted, each analysis consisted of four chains, random starting trees and the appropriate model (GTR+I+G) determined by Modeltest version 3.06. The chains were run for 2×10^5 generations with trees being sampled every 100 generations, stationary was determined visually, burn-in trees discarded and the remaining trees used to estimate Bayesian posterior probabilities.

2 Results

2.1 Sequences and variations of the mitochondrial 16S rRNA gene

In this study, the length of the mitochondrial 16S rRNA gene ranged from 1144 bp to 1341 bp in 25 taxa. Of the 1148 aligned sequences, 402 were variable and 242 were parsimony informative. The average proportions of the different nucleotide was T= 0.201, C= 0.23, A= 0.38, G= 0.19. These data clearly show that the base composition is somewhat A-rich and the base pair composition is rich in A and T bases (58.1%). An overall transitions/transversions ratio observed was 2.1 for these data sets. The saturation of transition and transversion changes was checked by plotting the percentage of sequence divergence against the pairwise number of inferred substitutions. The transitions/transversions ratio did not exhibit any plateau and indicated a non-mutational saturation (data not shown). The uncorrected *p*-distance matrix observed from the analysis of the alignment of all sequences is also not shown here, and the value of pairwise distance among labeonine fishes ranged from 0.1% to 16%, and from 14.8% to 20.4% between ingroups and outgroup.

2.2 Phylogenetic relationships

Bayesian analysis resulted in a consensus tree with great posterior probabilities at most nodes (Fig. 1). Within the Barbini species studied, two major clades could be recognized with great posterior probabilities (PP=98): one is composed of the subfamily Barbininae (*Onychostoma sima* and *Spinibarbus hollandi*), Schizothoracinae (*Schizothorax waltoni* and *Schizothorax moleworthi*) and Cyprininae (*Cyprinus multitaeniate*, *Cyprinus carpio*, *Carassius auratus* and *Procypris rabandi*) (PP=79). While the other comprises labeonine fishes with great posterior probabilities (PP= 100). The Labeoninae species is also related to two major groups: one includes genera *Cirrhinus*, *Crossocheilus* and *Garra*, and the other accommodates genera *Labeo*, *Sinilabeo*, *Osteochilus*, *Pseudocrossocheilus*, *Parasinilabeo*, *Ptychiodio*, *Semilabeo*, *Pseudogyrinocheilus*, *Rectoris* and *Discogobio*. In this latter association, the genera *Pseudogyrinocheilus*, *Rectoris* and *Ptychiodio* are closely related, and *Pseudocrossocheilus*, *Parasinilabeo* and *Semilabeo* closely related. They are related as the sister group of the genus *Discogobio*. Trees constructed using ML and Bayesian methods are very similar, maximum likelihood analysis yielded one tree with likelihood $\ln L=-6167.42083$ (not shown).

Maximum parsimony analysis yielded two most-parsimonious trees with a tree length= 1039, CI= 0.5600 and RI= 0.5733 (Fig. 2). In the MP tree, the genera *Sinilabeo*, *Osteochilus*, *Cirrhinus*, *Crossocheilus* and *Garra* form one clade; the genera *Pseudocrossocheilus*, *Parasinilabeo*, *Ptychiodio*,

Semilabe, *Pseudogyrinocheilus*, *Rectoris* and *Discogobio* are related as monophyletic with a strongly supported value of BP=80. The genus *Labeo* is basal in the labeonine fishes.

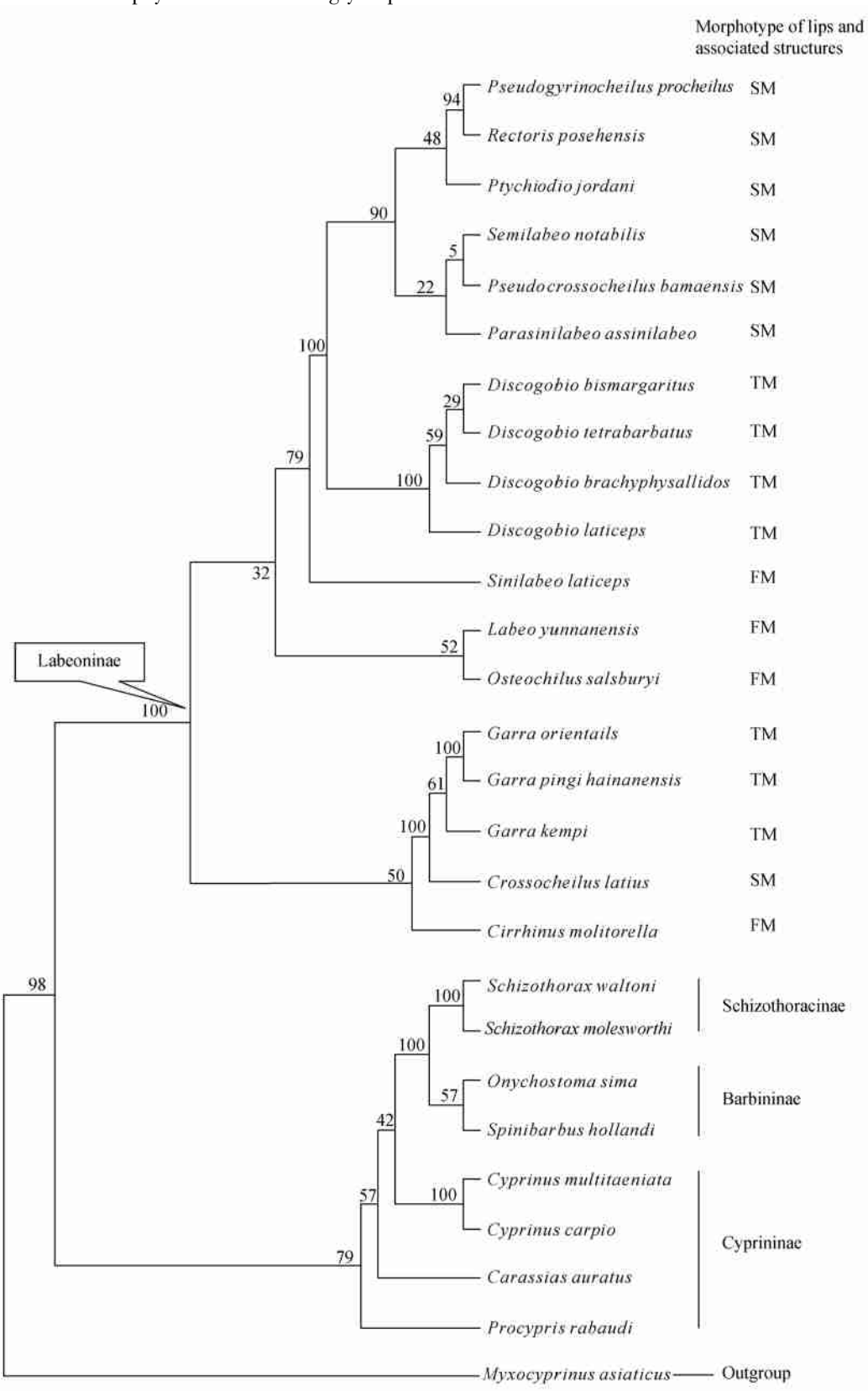

Fig. 1. Consensus tree obtained from Bayesian methods. Numbers at the nodes represent the posterior probabilities of the phylogeny, which was determined from the following 1001 trees. FM, the first morphotype; SM, the second morphotype; TM, the third morphotype.

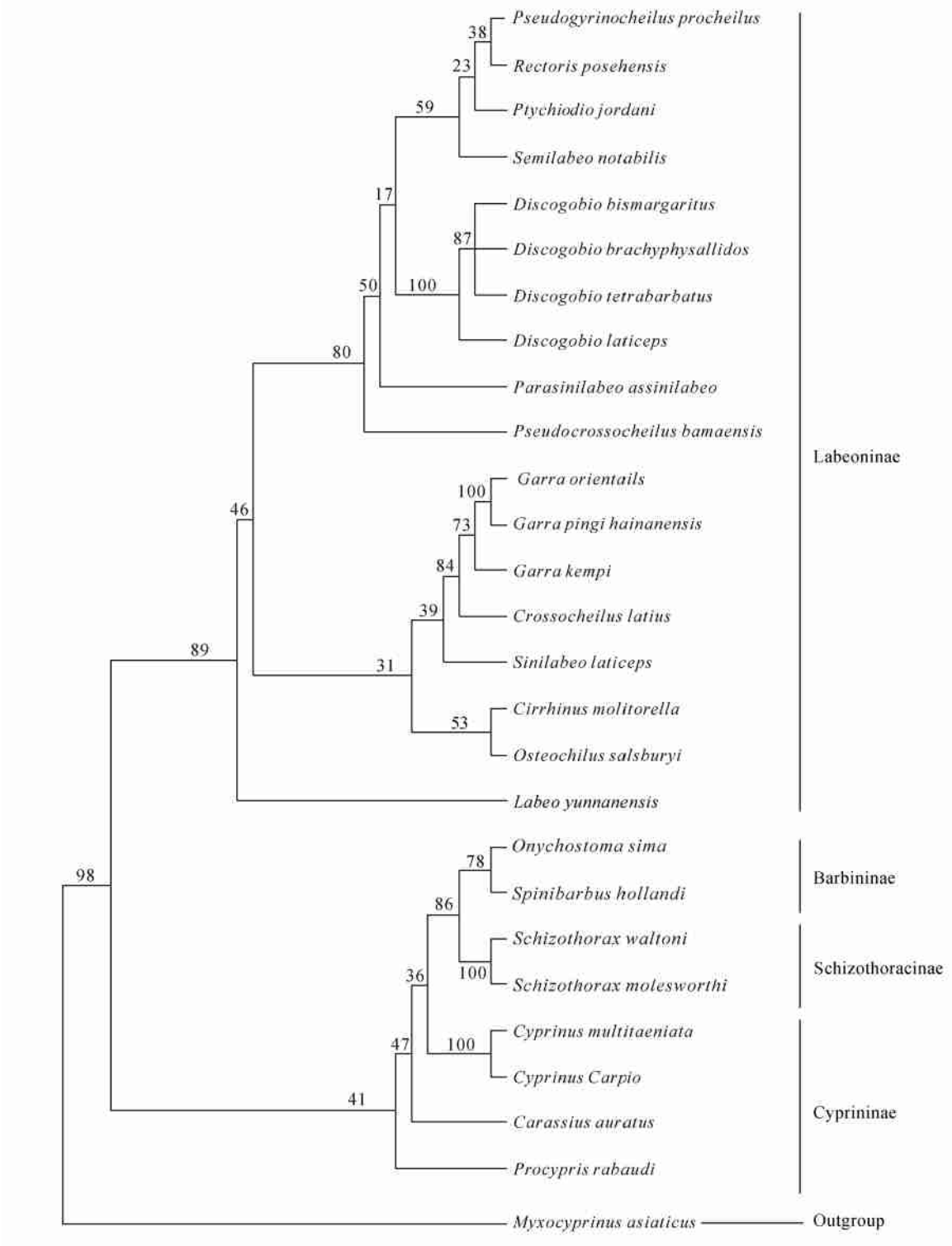

Fig. 2. Consensus tree of the two equiparsimonious trees inferred from maximum parsimony methods based on the mitochondrial 16S rRNA gene sequences. Length of each tree is 1039 steps and CI= 0.5600, RI= 0.5733, numbers at the nodes refer to the bootstrap.

The NJ tree is of the same topology with the MP tree for the labeonine species except for the position of the genus *Labeo*. In the NJ tree, *Labeo* is related as the sister group with the group including the genera *Pseudogyrinocheilus*, *Rectoris*, *Ptychiodio*, *Semilabeo*, *Pseudocrossocheilus*, *Parasinilabeo* and *Discogobio*.

Overall, trees constructed using NJ, MP, ML and Bayesian methods show that labeonine fishes are

related closely as a monophyly.

3 Discussions

3.1 Monophyly of the subfamily Labeoninae

On the basis of several morphological synapomorphies, the labeonine fishes were defined as monophyletic group[5, 21]. When *Myxocyprinus asiaticus* was assigned as the outgroup, our present study suggested that Labeoninae is basically a monophyletic group and related as sister group with non-labeonine fishes in Barbininae, Cyprininae and Schizothoracinae.

3.2 Phylogenetic relationships

The morphology of lips and associated structures have been used to analyze the phylogeny of Labeoninae[22], and the subfamily Labeoninae is divided into three major groups[1]. The first group found is that the upper lip is present and rostral cap plus lower lip can not form a prebuccal cavity. It consists of the genera *Sinilabeo*, *Henicorhynochus*, *Lobocheilus*, *Labeo*, *Cirrhinus*, *Labiobarbus* and *Osteocheilus*. In this group, it was thought that the genera *Sinilabeo* and *Lobocheilus* were the sister group which was closely related with *Labeo* and *Cirrhinus*[22]. However, the phylogeny based on the mitochondrial 16S rRNA gene shows that the genus *Sinilabeo* is not clustered with the genera *Lobocheilus*, *Labeo* and *Cirrhinus*, whereas it is closely related with the genera *Parasinilabeo*, *Discogobio* and *Rectoris*. Surprisingly, the positioning of the genera *Labeo* and *Osteocheilus* was problematic and constitutes the main source of differences between the MP tree presented in Fig. 2 and trees obtained from the NJ, ML and Bayesian methods with the low bootstrap support.

The second group, characterized by a prebuccal cavity, has been considered including the genera *Crossocheilus*, *Epalzeorhynchos*, *Rectoris*, *Pseudocrossocheilus*, *Parasinilabeo*, *Semilabeo*, *Pseudogyrinocheilus*, *Ptychiodio* and *Sinocrossocheilus*[23], and *Parasinilabeo*-like fishes are a group endemic to China and composed of the genera *Parasinilabeo*, *Semilabeo* and *Pseudogyrinocheilus*. Zhang defined *parasinilabeo*-like fishes as a monophyletic group for the possession of four synapomorphies[24]. However, our study shows that *parasinilabeo*-like group is maybe a paraphyletic or polyphyletic group. Within this group, two well-supported clades are presented. The first clade contains the genera *Pseudogyrinocheilus*, *Rectoris* and *Ptychiodio*, there is a strong support for a sister group relationship between the genera *Pseudogyrinocheilus* and *Rectoris* (PP=94); the second clade includes the genera *Pseudocrossocheilus*, *Parasinilabeo* and *Semilabeo* with low bootstrap support. The genus *Pseudocrossocheilus* endemic to China had been regarded as a synonym of the genus *Crossocheilus*[3, 22]. Zhang made a taxonomical revision of the Chinese cyprinid genus *Crossocheilus* and considered the genus *Pseudocrossocheilus* as a valid genus[6]. In this study, the genus *Pseudocrossocheilus* is not clustered with the genus *Crossocheilus*, however, it has a closer relationship with *parasinilabeo*-like fishes.

The third group, defined by the presence of the adhesive disc on the chin and pharyngeal teeth in 2 rows includes the genera *Garra*, *Discogobio*, *Placochilus* and *Discocheilus*. Wu et al. considered that the garra-like group, composed of the genera *Garra*, *Discogobio* and *Placochilus*, might be the most derived group[22]. The studies on development, function and surface ultrastructure of the adhesive disc also supported that the genus *Garra* had a close relationship with genus *Discogobio*[25]. However, our phylogenetic analysis indicates that *Garra* has a close relationship with *Corrocheilus* and *Cirrocheilus*, while it is distantly related with the genus *Discogobio*. The garra-like group might be a paraphyletic or polyphyletic group, because the genus *Garra* is clustered with the first group, while the genus *Discogobio* has a close relationship with the second group. The adhesive disc on the chin is only a feature adapted to torrential environment and of little significance in the phylogeny of the subfamily Labeoninae[26, 27].

In Labeoninae, modifications of the lips and associated structures are an indication for adaptive evolution to lotic systems. These characters, as mentioned above, were used as traditional techniques to elucidate the relationships of labeonine fishes. In the present paper we present an inconsistent phylogeny based on the analysis of sequence data of the mitochondrial 16S rRNA gene. Our phylogenetic analysis indicates that Labeoninae may be divided into 2 major clades: one comprising the genera *Cirrhinus*, *Crossocheilus* and *Garra*, and the other consisting of the genera *Labeo*, *Sinilabeo*, *Osteochilus*, *Pseudocrossocheilus*, *Parasinilabeo*, *Ptychiodio*, *Semilabeo*, *Pseudogyrinocheilus*, *Rectori* and *Discogobio*.

3.3 Relationships within Barbini Series

Morphological and molecular analyses supported the Barbini Series including the subfamily Barbininae, Labeoninae, Cyprininae and Schizothoriacinae to be a monophyly[1, 5, 28]. Based on morphological traits and fossil record, Chen et al. considered that Barbininae was the primitive subfamily and was more closely related to Labeoninae than to Cyprininae, and Labeoninae might derive from the common ancestor of the Barbininae[1]. The present work proposes that subfamily Barbininae, Labeoninae and Schizothoracinae form a monophyletic group respectively, whereas monophyly of the subfamily Cyprininae is poorly resolved. Our phylogenetic analysis also suggests a sister group relationship between the subfamily Labeoninae and a group containing Barbininae, Cyprininae and Schizothoracinae.

Results in this paper provide a good start towards understanding Labeoninae relationships. However, this study include neither all representatives of this monophyletic subfamily in China nor any of the diversity present in the African species. At the same time, intergeneric and intrageneric relationships within those four subfamilies are far from being univocal. Therefore, further morphological and molecular data will serve to clarify the relationships among members' representative cyprinids taxa of the Barbini Series at the species level.

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