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Molecular phylogeny of *Sinocyclocheilus* (Cypriniformes: Cyprinidae) inferred from mitochondrial DNA sequences

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Abstract

More than 10 species within the freshwater fish genus *Sinoncyclocheilus* adapt to caves and show different degrees of degeneration of eyes and pigmentation. Therefore, this genus can be useful for studying evolutionary developmental mechanisms, role of natural selection and adaptation in cave animals. To better understand these processes, it is indispensable to have background knowledge about phylogenetic relationships of surface and cave species within this genus. To investigate phylogenetic relationships among species within this genus, we determined nucleotide sequences of complete mitochondrial cytochrome *b* gene (1140 bp) and partial ND4 gene (1032 bp) of 31 recognized ingroup species and one outgroup species *Barbodes laticeps*. Phylogenetic trees were reconstructed using maximum parsimony, Bayesian, and maximum likelihood analyses. Our phylogenetic results showed that all species except for two surface species *S. jii* and *S. macrolepis* clustered as five major monophyletic clades (I, II, III, IV, and V) with strong supports. *S. jii* was the most basal species in all analyses, but the position of *S. macrolepis* was not resolved. The cave species were polyphyletic and occurred in these five major clades. Our results indicate that adaptation to cave environments has occurred multiple times during the evolutionary history of *Sinocyclocheilus*. The branching orders among the clades I, II, III, and IV were not resolved, and this might be due to early rapid radiation in *Sinocyclocheilus*. All species distributed in Yunnan except for *S. rhinocerous* and *S. hyalinus* formed a strongly supported monophyletic group (clade V), probably reflecting their common origins. This result suggested that the diversification of *Sinocyclocheilus* in Yunnan may correlate with the uplifting of Yunnan Plateau.
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Keywords: *Sinocyclocheilus*; Mitochondrial DNA; Phylogeny; Cave; Rapid radiation

1. Introduction

Cave animals have attracted much attention for their distinct troglomorphic characters, including the enlargement of some sensory organs and appendages, and the reduction and/or loss of eyes and pigmentation (Culver et al., 1995; Romero, 2001). Main focuses on the evolution of cave animals involve in the process of cave colonization and the mechanisms underlying these troglomorphic characters (Dowling et al., 2002). Most studies utilize *Astyanax* cavefish populations as model organisms for understanding how these animals and their troglomorphic features have evolved. It has been demonstrated that different *Astyanax* cavefish populations with different degree of eye and pigment degeneration may have evolved the similar eyeless phenotype independently

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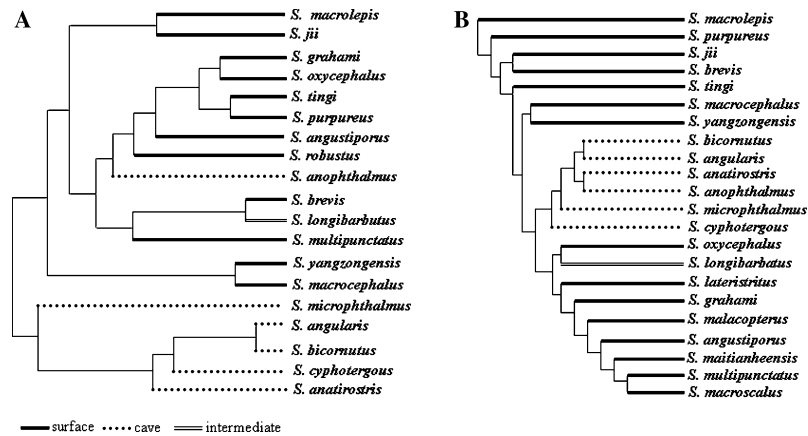[1] These authors contributed equally to this work.

Fig. 1. Previous phylogenetic hypotheses based on morphological data: (A) phenetic relationships of 19 species of *Sinocyclocheilus* inferred from 30 characters (Shan and Yue, 1994), (B) cladistic relationships of 22 species of *Sinocyclocheilus* from 28 characters (Wang et al., 1999). Drawings are not to scale.

(Dowling et al., 2002; Jeffery et al., 2003; Mitchell et al., 1977; Strecker et al., 2003; Strecker et al., 2004; Wilkens, 1988; Wilkens and Strecker, 2003; Yamamoto et al., 2003). Genetic and developmental studies indicate that troglomorphic traits are controlled by multiple regulatory genes (Borowsky and Wilkens, 2002; Jeffery, 2001; Wilkens, 1988; Yamamoto et al., 2004). These studies make cavefish as an ideal model system in evolutionary developmental biology (Jeffery, 2001). In addition, extensive researches on cave-dwelling crustacean, *Gammarus minus*, have revealed that cave animals can provide valuable empirical models for the study the role of natural selection and adaptation in evolution (Culver et al., 1995).

Recent progresses on cave animals have been mainly made in a single cave species, such as *Astyanax mexicanus* or *Gammarus minus*. This is probably ascribed to the lack of good model systems, in which several closely related species adapted to caves possess similar phenotypes. One promising exception is the freshwater fish genus *Sinocyclocheilus* (Family: Cyprinidae, subfamily: Barbinae), which is endemic to China, and distributes in karst cave waters and surface rivers or lakes in Yungui Plateau (including Yunnan Province and Guizhou Province) and its surrounding region (Guangxi Zhuang Autonomous Region) (Shan et al., 2000). Currently, this genus includes over 30 species. All these species live in two different habitat types, karst cave waters and surface rivers or lakes. A few of them are intermediate species—those surface species that sometimes live in cave waters (Chen and Yang, 1993; Wang and Chen, 1989). Because cave habitats are characterized by permanent darkness, absence of green plants, and food shortage (Chen and Yang, 1993; Chen et al., 1994; Poulson and White, 1969), these cave species have acquired some troglomorphic traits in adaptation to these environments, including different degree of eye and pigment degeneration and well-developed projection of frontal and parietal bones.

It is not clear whether different cave species within *Sinocyclocheilus* have a common origin. Morphological results of Shan and Yue (1994) (Fig. 1A) showed that all species in their study were grouped into two monophyletic clusters, which included all surface species and all cave species, respectively, except one cave species *S. anophthalmus*. Their results suggested that all cave species had two independent recent common ancestors. However, the morphological phylogenetic results of Wang et al. (1999) (Fig. 1B) showed that all cave species formed a monophyletic subclade, which nested in surface species. Their results revealed that all cave species had a common origin. Therefore, those hypotheses for the origin of cave species within *Sinocyclocheilus* need to be tested with independent evidence.

The genus *Sinocyclocheilus* can be useful for studying evolutionary developmental mechanisms and role of natural selection and adaptation in cave animals. To better understand these processes, it is indispensable to have background knowledge about phylogenetic relationships of surface and cave species within this genus. So far, phylogenetic relationships within *Sinocyclocheilus* remain poorly understood. Here we use mitochondrial cytochrome *b* (Cyt *b*) and NADH dehydrogenase subunit 4 (ND4) gene sequences to reconstruct the phylogenetic relationships of *Sinocyclocheilus*. The purposes of this study are to (1) reconstruct the phylogeny of genus *Sinocyclocheilus*, (2) understand the origin of cave species, and (3) discuss the historical biogeography of *Sinocyclocheilus* in context of its molecular phylogeny.

2. Materials and methods

2.1. Sample collection

A total of 56 specimens of 31 recognized species of the genus *Sinocyclocheilus* were examined (see Table 1). Our

Table 1
Species names, sampling localities, river drainage, sample size, and haplotype numbers

Species	Locality	River drainage	Sample	Haplotype
Sinocyclocheilus macrocephalus	Heilongtan, Shilin County, Yunnan	Nanpanjiang	2	2
Sinocyclocheilus oxycephalus	Heilongtan, Shilin County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus lunanensis	Heilongtan, Shilin County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus maitianheensis	Jiuxiang,Yiliang County,Yunnan	Nanpanjiang	1	1
Sinocyclocheilus anophthalmus	Jiuxiang,Yiliang County,Yunnan	Nanpanjiang	2	1
Sinocyclocheilus malacopterus	Jinji, Luoping County, Yunnan	Nanpanjiang	2	1
	Wulonghe, Shizong County, Yunnan	Nanpanjiang	1	1
	Changdi, Luoping County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus guishanensis	Guishan, Shilin County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus yangzongensis	Yangzonghai Lake, Yunnan	Nanpanjiang	2	2
Sinocyclocheilus qujingensis	Wujiafen, Qujing, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus rhinocerous	Luoping County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus angustiporus	Luxi County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus hyalinus	Alugudong, Luxi County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus huaningensis	Huaning County, Yunnan	Nanpanjiang	1	1
Sinocyclocheilus lateristritus	Luliang County, Yunnan	Nanpanjiang	1	1
	Maojiachong, Zhanyi County, Yunnan	Beipanjiang	2	2
	Shannabian, Zhanyi County, Yunnan	Beipanjiang	1	1
	Luoshuidong, Zhanyi County, Yunnan	Beipanjiang	1	1
Sinocyclocheilus tingi	Fuxianhu Lake, Yunnan	Nanpanjiang	2	1
Sinocyclocheilus grahami	Qinglongsi, Kunming, Yunnan	Jinshajiang	1	1
	Huanglongdong, Kunming, Yunnan	Jinshajiang	1	1
	Haikou, Kunming, Yunnan	Jinshajiang	1	1
Sinocyclocheilus microphthalmus	Shadong, Lingyun County, Guangxi	Hongshuihe	2	2
	Luolou, Lingyun County, Guangxi	Hongshuihe	2	1
Sinocyclocheilus lingyunensis	Shadong, Lingyun County, Guangxi	Hongshuihe	2	2
	Jiangcun, Lingyun County, Guangxi	Hongshuihe	1	1
Sinocyclocheilus anatirostris	Leye County, Guangxi	Hongshuihe	1	1
Sinocyclocheilus tianeensis	Banshi, Tiane County, Guangxi	Hongshuihe	1	1
Sinocyclocheilus furcodorsalis	Bala, Tiane County, Guangxi	Hongshuihe	1	1
Sinocyclocheilus halfibindus	Fengshan County, Guangxi	Hongshuihe	1	1
Sinocyclocheilus altishoulderus	Mashan County, Guangxi	Hongshuihe	1	1
Sinocyclocheilus jii	Fuchuan County, Guangxi	Hejiang	2	2
Sinocyclocheilus macrolepis	Nandan County, Guangxi	Dagouhe	1	1
Sinocyclocheilus macrophthalmus	Xiaao, Duan County, Guangxi	Hongshuihe	3	1
Sinocyclocheilus jiuxuensis	Jiuxu, Hechi, Guangxi	Hongshuihe	2	1
Sinocyclocheilus bicornutus	Xingren County, Guizhou	Beipanjiang	3	1
Sinocyclocheilus cyphotergous	Luodian County, Guizhou	Hongshuihe	1	1
Sinocyclocheilus multipunctatus	Huishui County, Guizhou	Hongshuihe	2	2
Sinocyclocheilus longibarbatus	Libo County, Guozhou	Dagouhe	1	1
Barbodes laticeps	Zhenning County, Guizhou	Beipanjiang	2	1

samples covered main distribution ranges of *Sinocyclocheilus* species, including Yunan Province (Yunnan), Guizhou Province (Guizhou), and Guangxi Zhuang Autonomous Region (Guangxi) (Fig. 2). Two individuals of *Barbodes laticeps* belonging to the genus *Barbodes* were chosen as outgroup in the light of current knowledge of the phylogenetic relationships among fishes within subfamily Barbinae (Wu et al., 1977). Muscle tissues were maintained in 90% ethanol. Voucher specimens are deposited in Animal Museum of Department of Biology, Yunnan University, Kunming, China.

2.2. DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from muscle tissues by standard phenol–chloroform extraction techniques (Sambrook et al., 1989). Complete sequences of Cyt *b* gene and fragments of ND4 gene were amplified using polymerase chain reaction with primers in Table 2. PCRs were performed in a total volumes of 50 μl and contained 5 μl 10 × reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM each primer, 1.5 U *Taq* DNA polymerase (TaKaRa Biosystems), and approximately 30 ng genomic DNA. Amplification was implemented with denaturing at 95 °C for 3 min, 35 cycles of denaturing at 94 °C for 50 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min, followed by extension at 72 °C for 5 min. Double-stranded amplified product was electrophoresed in a 1.5% agarose gel and successful amplifications were excised from the gel and purified by Watson PCR Purification Kit (Watson BioTechnologies, Shanghai) according to manufacturer's instructions. The
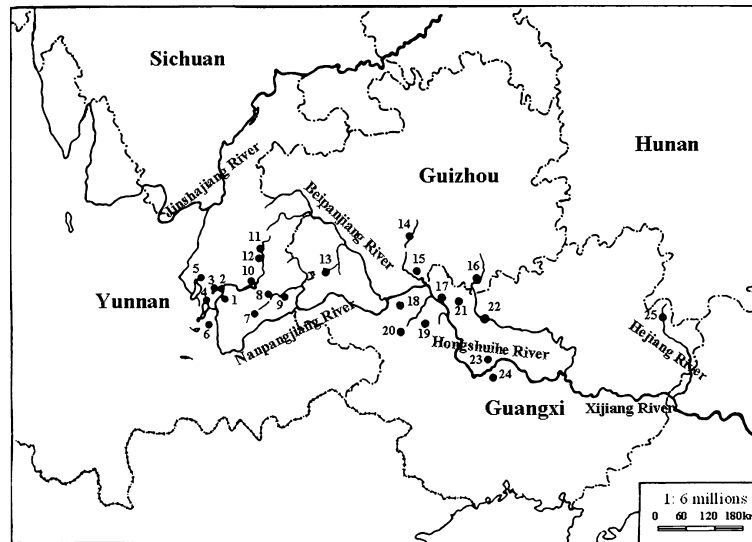
Fig. 2. The distribution of the *Sinocyclocheilus* species. The numbers on the map indicate sample localities in Yunnan Province, Guizhou Province, and Guangxi Zhuang Autonomous Region, respectively. Yunnan Province including: (1) Shilin, (2) Yiliang, (3) Yangzonghai, (4) Chengjiang, (5) Kunming, (6) Huaning, (7) Luxi, (8) Shizong, (9) Luoping, (10) Luliang, (11) Zhanyi, and (12) Qujing; Guizhou Province including: (13) Xingren, (14) Huishui, (15) Luodian, (16) Libo; Guangxi Zhuang Autonomous Region including: (17) Tiane, (18) Leye, (19) Fengshan, (20) Lingyun, (21) Nandan, (22) Hechi, (23) Duan, (24) Mashan, and (25) Fuchuan.

Table 2
List of primers used in this study

Gene	Primer	Sequences	Reference
Cyt *b*	L14737	5′-CCA CCG TTG TTA ATT CAA CTA C-3′	This study
	L15519	5′-GGA GAC CCA GAA AAC TTT ACC CC-3′	Xiao et al. (2001)
	H15374	5′-AGA TTT TGT CTG CGT CTG AG-3′	This study
	H15492	5′-GGG GTG AAG TTT TCT GGG TC-3′	This study
	L15286	5′-ACA CGA TTC TTC GCA TTC CAC-3′	This study
	H15915	5′-CTC CGA TCT CCG GAT TAC AAG AC-3′	Xiao et al. (2001)
ND4	L11264	5′-ACG GGA CTG AGC GAT TAC-3′	This study
	H11900	5′-CCG TAT AGT GGT ATT TTA AC-3′	This study
	L11835	5′-TTA GTA CTC CAA TAT TCA CA-3′	This study
	L11857	5′-TGA GGC CAC ATA ATC TGA TGA GC-3′	This study
	H12320	5′-AAT CAT TTG TAG TCC TCG GGC GAG-3′	This study
	H12346	5′-TCA TCA TAT TGC GGT TAG-3′	This study

Note. The position of 3′ end oligonucleotide of each primer is given relative to the published sequence of human mtDNA (Anderson et al., 1981) for cytochrome *b* and of *Cyprinus carpio* mtDNA (Chang et al., 1994) for ND4, respectively.

purified product was cyclesequenced in both forward and reverse directions using the same primers for PCR amplification with the ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit in 5 µl volumes (Applied Biosystems). The cycling profile for the sequencing reaction consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Isopropyl alcohol-purified cyclesequencing products were analyzed on the ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

DNA sequences were edited and aligned using DNA-STAR5.0 (DNASTAR Inc.) and checked manually as well. The Cyt *b* and the ND4 genes were aligned based on the putative amino acid sequence.

2.3. Phylogenetic analyses

Aligned sequence data were imported into MEGA 2.1 (Kumar et al., 2001) for analyses of nucleotide composition. Nucleotide saturation was analyzed by plotting the number of transitions and transversions on each codon position against the Tamura and Nei (1993) (TN93) genetic distance using DAMBE program (Xia and Xie, 2001). Because transitions and transversions in the two mitochondrial Cyt *b* and ND4 genes were accumulated linearly and showed no saturation patterns at any position in each gene analyzed (plots not shown), so all nucleotide positions were employed in subsequent analysis.

Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2003). For all phylogenetic analyses, *Barbodes laticeps* was used as outgroup. Data from the two mitochondrial protein-coding genes were initially analyzed separately with maximum parsimony (MP) method. To examine possible incongruence between these two genes, we used an incongruence length difference (ILD) test (Farris et al., 1994, 1995) referred to as a partition homogeneity test in PAUP*. We implemented 1000 replicates of the ILD test with 100 random addition sequences. The test revealed significant incongruence between these two genes ($P = 0.001$), however, we still prefer the combination of the two genes for the following reasons. First, more and more studies indicated that multiple data sets can be combined even if incongruence were detected (Cunningham, 1997; Darlu and Lecointre, 2002; Yoder et al., 2001). Second, given that the entire animal mitochondrial genome is inherited as a single unit without recombination (Moore, 1995; Nei, 1991; Page, 2000), it is logical to combine and analyze the two genes as a single data. Thus, we also conducted a MP analysis for the combined data set. Each nucleotide was treated as an unordered character with four alternative states. Most parsimonious trees (MPTs) were generated using heuristic search routines with 100 random addition sequences and tree-bisection-reconnection (TBR) branch swapping. Support for nodes was assessed using non-parametric bootstrapping (Felsenstein, 1985) with 1000 replications with the same search options as mentioned above. A node was interpreted as strongly supported if the bootstrap percentage (BP) was $\geqslant 70\%$ (Hillis and Bull, 1993).

In a Bayesian analysis (Huelsenbeck et al., 2000; Larget and Simon, 1999) with MrBayes 3.04 (Huelsenbeck and Ronquist, 2001), the combined data set was treated as two partitions with different models accounted for their heterogeneity. The prior models of sequence evolution employed during Bayesian analysis for both Cyt *b* and ND4 data sets were determined using Modeltest 3.06 (Posada and Crandall, 1998) based on the likelihood ratio tests. The tests indicated that HKY + I + Γ and TrN + I + Γ models were the most appropriate models for the two data sets, respectively. We utilized the "unlink" command in MrBayes 3.04 to unlink the following parameters: "unlink shape = (all) pinvar = (all) statefreq = (all) revmat = (all)." Random starting trees were used, and analyses were run for 2.0×10^6 generations, sampling the Markov chains at intervals of 100 generations thinned the data to 20,000 sample points. We ran two independent analyses starting from different random trees to assure that our analyses were not trapped in local optima. All sample points prior to reaching convergence (2000 trees) were discarded as burn-in samples. The remaining samples were used to generate a majority rule consensus tree, where percent-

age of samples recovering any particular clade represented the clade's posterior probability (PP) (Huelsenbeck and Ronquist, 2001). Probability $\geqslant 95\%$ were considered indicative of significant support (Reeder, 2003; Zkharov et al., 2004).

In addition, maximum likelihood (ML) analysis of the combined data was performed under the TrN + I + Γ model selected by Modeltest 3.06 (Posada and Crandall, 1998) based on the likelihood ratio tests. Settings for the TrN + I + G model were as follows: *R*-matrix (1.0000, 32.1990, 1.0000, 1.0000, and 17.4637); base frequencies (A 0.3227, C 0.2939, G 0.1197, and T 0.2637); proportion of invariant sites 0.5266; and the shape parameter of the gamma distribution 1.1408. A heuristic ML search with 10 random additional sequences and TBR branch swapping was performed with this model. Branch support for the ML tree was assessed using non-parametric bootstrapping (Felsenstein, 1985) with 100 heuristic replicates with single random addition replicates.

A Bayesian analysis of combined data was also used to estimate the divergence times for major clades by software BEAST v1.1.2 (Drummond and Rambaut, 2003). We first ran MCMC for 1.0×10^6 steps sampling every 100 under GTR + I + Γ model. After adjusting the parameter settings based on recommendations of the first run, a second run with longer chain 2.0×10^6 steps was performed using the same model. According to the tutorials of BEAST, the effective sample sizes (ESSs) of parameters sampled from MCMC in our run were more than 200. The results were viewed using the software Tracer v1.1.1 (Rambaut and Drummond, 2003).

3. Results

3.1. Sequences characteristics

All sequences are deposited in GenBank (Accession Nos. AY854683-AY854796). The complete Cyt *b* and fragments of ND4 sequences analyzed were 1140 and 1032 bp in length, respectively. Neither of the two protein-coding genes had premature stop codons or ambiguous nucleotide in translation, indicating that these sequences were functional genes. Since some sequences from the same taxa were identical, those taxa in all subsequent analyses were reduced to one representative per taxon (shown in Table 1), yielding a data set of 48 sequences including outgroup.

The molecular characteristics of the Cyt *b* and ND4 genes were presented in Table 3. Nucleotide base composition showed low level of G (14.6% across all sites all taxa and only 6.3% at the third position) (Table 3) which is characteristic for the mitochondrial genome (Cantatore et al., 1994).

Table 3
Molecular characteristics of the mitochondrial Cyt *b* and ND4 genes for the 48 sequences analyzed

	Cyt *b*	ND4	Combined data
Nucleotide composition (%)			
A	29.7	31.1	30.3
C	27.9	27.2	27.6
G	14.6	14.6	14.6
T	27.8	27.2	27.5
Variable sites			
First codon position	101 (8.86%)	70 (6.78%)	171 (7.87)
Second codon position	33 (2.89)	23 (2.23)	56 (2.58%)
Third codon position	315 (27.63%)	280 (27.13%)	595 (27.39%)
Total	449 (39.39)	373 (36.14%)	822 (37.85%)
Phylogenetically informative sites			
First codon position	78 (6.84%)	52 (5.04%)	130 (5.99%)
Second codon position	16 (1.40%)	12 (1.16%)	28 (1.29%)
Third codon position	271 (23.77%)	243 (23.55%)	514 (23.66%)
Total	365 (32.02%)	307 (29.75%)	672 (30.94%)
Ti/Tv ratio	6.8	11.6	7.8

3.2. Phylogenetic analyses

Parsimony analysis using equal weights resulted in 16 MPTs (length, 1299; CI, 0.434; RI, 0.777; and RC, 0.338) for Cyt *b* data set and 10 MPTs (length, 1011; CI, 0.445; RI, 0.766; and RC, 0.341) for ND4 data set. The strict consensus trees of these two separate data sets (not shown) were largely identical in interspecific relationships, except for minor incongruence among some deep branch orders. Notably, the non-parametric bootstrap values on these branches were low (BP < 50, not shown). The unweighted parsimony analysis of the combined data set generated 24 equally MPTs (length, 2343; CI, 0.433; RI, 0.767; and RC, 0.332). The strict consensus tree of these 24 trees (Fig. 3) was mostly congruent with those of separate data sets but with higher bootstrap values for most branches. Within the genus *Sinocyclocheilus*, *S. jii* was placed as the most basal branch with strong support and *S. macrolepis* was the next most basal lineage with moderate support (BP = 56). The remaining species formed five strongly supported major clades (I, II, III, IV, and V), but the relationships among clades I, II, III, and IV were not resolved as indicated by bootstrap values in Fig. 3.

The topology of Bayesian tree of combined data set (Fig. 4) was completely identical with that of ML tree of combined data set (−ln *L* = 13779.25). The five major well-supported clades in MP tree of combined data were recovered in Bayesian and ML trees with strong support. All phylogenetic analyses strongly supported the most basal position of *S. jii*. The differences between Bayesian and ML trees and MP tree were the position of *S. macrolepis* and the branch orders among clades I, II, III, and IV. In addition, internal sub-branch orders in the clade V also differed between Bayesian and ML trees and MP tree. Notably, all these topological differences were

weakly supported (BP < 50, PP < 0.95), but the interspecific relationships within *Sinocyclocheilus* were almost identical between analyses with strong support.

Since there is no fossil record in *Sinocyclocheilus* fishes, the molecular clock calibration rates of 0.8–2.6% per million years of fish mitochondrial protein-coding genes (Mckay et al., 1996; Orti et al., 1994; Taylor and Dodson, 1994) were used to estimate the divergence times of major clades. The divergence dates for the major five clades I, II, III, IV, and V at 95% highest posterior density (HPD) interval were 0.182–0.588, 0.63–2.05, 2.524–8.177, 1.94–6.299, and 2.453–7.928 million years ago, respectively.

4. Discussion

4.1. Phylogenetic relationships within Sinocyclocheilus

All our phylogenetic analyses showed that all species clustered as five major monophyletic clades with strong supports (Figs. 3 and 4), except for two surface species *S. jii* and *S. macrolepis*. All phylogenetic results strongly agreed with the most basal position of *S. jii*. This result was not in conflict with previous morphological studies, where *S. jii* exhibited more traits similar to the outgroup *Barbodes* fishes (Shan and Yue, 1994; Wang et al., 1999). The relationship of *S. macrolepis* to other species was not resolved because of its unstable position among the gene trees.

Our phylogenetic results resemble little to those of previous studies. Morphological result of Shan and Yue (1994) revealed that all species in their study clustered as two reciprocally monophyletic groups, in which one group included all surface species and one cave species *S. anophthalmus*, the other included all cave species
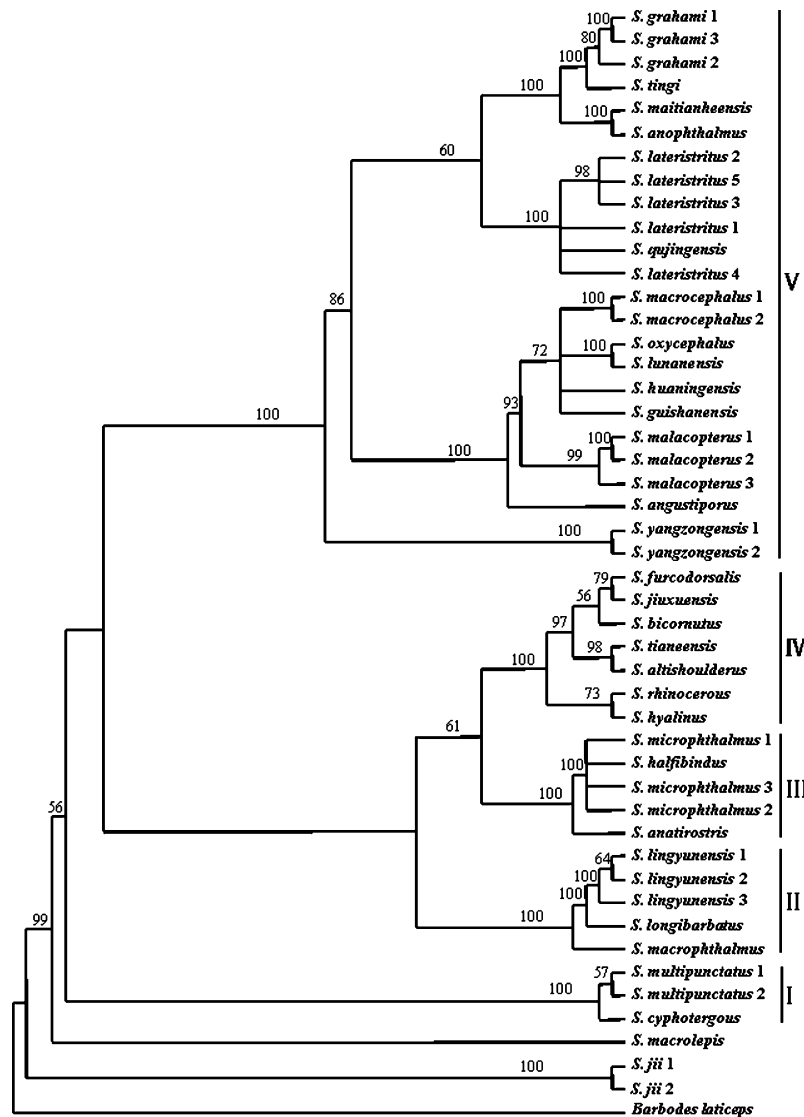
Fig. 3. The strict consensus tree generated by MP analysis based on the combined data. Numbers at the nodes denote the bootstrap percentages of 1000 replicates (only those ⩾50% are shown).

(shown in Fig. 1A). The distinctive morphological differences between these two groups were the variations of frontal, scale number, and radius. Species in the group containing all cave species shared the following characters: less scale numbers, scale radius only present in scale apical region, and acutely upheaved frontal–parietal area. Therefore, they regarded that these characters had phylogenetic significance and the genus *Sinocyclocheilus* could be subdivided into two subgenera, *Sinocyclocheilus* and *Gibbibarbus* corresponding to the two groups. Wang et al. (1999) presented a different phylogeny of this genus based on most osteological characters (Fig. 1B). Species of these two subgenera in Shan and Yue's tree (1994) were intermingled. In addition, Wang and Chen (1999) reexamined the distinguishing characters between the two subgenera and found that scale numbers within *Sinocyclocheilus* were continuous, and the difference in

scale radius between the two subgenera was probably due to convergence. Consequently, they did not support the view that this genus could be subdivided into two subgenera *Sinocyclocheilus* and *Gibbibarbus* (Wang and Chen, 1999). However, Wang et al. (1999) also treated the states of frontal–parietal area as one of phylogenetically informative characters. Thus, all cave species in their study clustered as a subclade and nested in surface species.

To investigate phylogenetic utilities of these characters, we mapped the character states of scale radius and frontal–parietal area onto our molecular phylogenetic tree (Fig. 4). The states of scale radius were available only for species in Shan and Yue's (1994) morphological study. The states of frontal–parietal area were based on previous two morphological studies and original literature. The results showed that the species
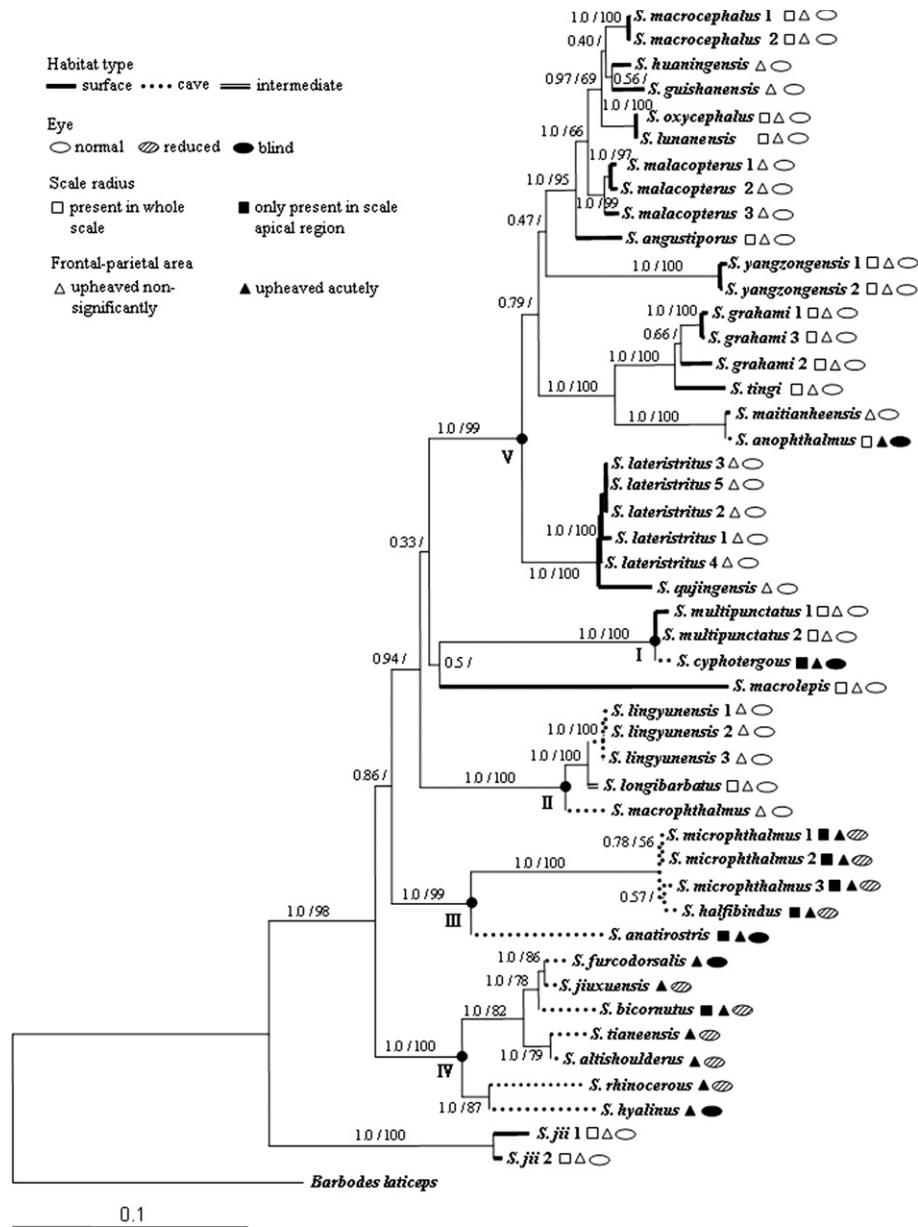
Fig. 4. Bayesian tree of combined data for each data partition with different models. Posterior probabilities followed by non-parametric bootstrap proportions with 100 replicates by using the fast stepwise-addition option for ML tree (only those ⩾50% are shown).

sharing same states of these two characters did not form a monophyletic group, but clustered with other species in different clades. For instance, *S. cyphotergous* and *S. bicornutus*, had the same character states (scale radius only present in scale apical region and acutely upheaved frontal–parietal area), but these two species were placed in clades I and IV, respectively. Therefore, the characters shared among cave species might be due to convergent evolution and were not phylogenetically informative. Just as Shan et al. (2000) pointed out that the strong selective pressures caused by cave environments had profound effect on character evolution and it was difficult to discriminate between convergence and shared traits due to common ances-

try, the incongruent phylogenetic relationships within *Sinocyclocheilus* between molecular and morphological results can mainly be attributed to convergence in morphological characters in cave species. Our results provided further evidence in support of the view that the *Sinocyclocheilus* was not suited to be subdivided into the subgenera *Sinocyclocheilus* and *Gibbibarbus* (Wang and Chen, 1999).

4.2. Troglomorphic adaptation and origin of cave species

During the evolution of cave fishes, reduction and/or loss of eyes and melanin pigmentation, an expanded gustatory system, and other troglomorphic traits have been

common phenomena (Jeffery, 2001; Jeffery and Martasian, 1998; Jeffery et al., 2000; Romero, 2001; Wilkens, 1988). Previous morphological studies on *Sinocyclocheilus* species revealed that the degree to which these troglomorphic traits occurred varied (Chen and Yang, 1993; Chen et al., 1994, 1997; Wang and Chen, 2000). For better understanding the evolution of troglomorphic adaptation, we mapped two major troglomorphic traits (the character states of eyes and frontal–parietal area) onto our molecular phylogenetic tree (Fig. 4). The states of these two traits for each species were based on the previous morphological studies and description of original literature. All surface species uniformly have normal eyes and non-significantly upheaved frontal–parietal area. Notably, the states of two troglomorphic traits in the two cave species *S. lingyunensis* and *S. macrophthalmus* are also same as those of surface species. In fact, *S. lingyunensis* and *S. macrophthalmus* were in the primary stage adaptive to cave environments and showed some trolomorphic traits, such as loss of melanin pigmentation (Li et al., 2000; Zhang and Zhao, 2001). Except for these two cave species, the remaining cave species all have acutely unheaved frontal–parietal area. As far as blind state of eyes was concerned, all cave species sharing this character state were placed in different major clades (*S. cyphotergous* in clade I, *S. anatirostris* in clade III, *S. furcodorsalis* and *S. hyalinus* in clade IV, and *S. anophthalmus* in clade V). The state of reduced eyes was observed in two species of clade III (*S. microphthalmus* and *S. halfibandus*) and five species in clade IV (*S. jiuxuensis*, *S. bicornutus*, *S. tianeensis*, *S. altishoulderus*, and *S. rhinocerous*). These results indicated that evolution of the two troglomorphic traits was correlated with cave environments.

Previous studies have demonstrated that different *Astyanax* cavefish populations with various degrees of eye degeneration may have evolved the eyeless phenotype independently (Dowling et al., 2002; Jeffery et al., 2003; Mitchell et al., 1977; Strecker et al., 2003, 2004; Wilkens, 1988; Wilkens and Strecker, 2003; Yamamoto et al., 2003). Our molecular phylogenetic results also revealed that different cavefish species within *Sinocyclocheilus* have invaded into cave waters multiple times and acquired their troglomorphic traits independently. It is fascinating and unique that there are so many cavefish species with varying degree of troglomorphic adaptations in a single fish genus, and this will make the genus *Sinocyclocheilus* as an excellent model system for studying convergent evolution (Romero and Paulson, 2001). Interestingly, our results revealed that two blind cavefish species *S. cyphotergous* and *S. anophthalmus* had sister-taxon relationship with the surface *S. multipunctatus* and *S. maitianheensis*, respectively. It will make these two cavefish species as additionally invaluable model systems in evolutionary developmental biology for studying the molecular mechanisms responsible for eyes degeneration

and other troglomorphic traits, as well as *Astyanax* cavefish populations (Jeffery, 2001; Jeffery and Martasian, 1998; Jeffery et al., 2000; Strickler et al., 2001; Yamamoto and Jeffery, 2000; Yamamoto et al., 2004).

4.3. Historical biogeography

In our phylogenetic trees, there was no resolution for the deeper branches (among clades I, II, III, and IV). Two possible causes could interpret it: rapid radiation of lineages and multiple hits (homoplasy) (Tsigenopoulos and Berrebi, 2000). However, among *Sinocyclocheilus* species, the results of nucleotide saturation analyses showed that there were no saturation patterns at any position in the two protein-coding genes (plots not shown). In addition, there was also no resolution among these five major clades in the separate and combined phylogenetic analyses of amino acid sequences of the two genes (results not shown). All analyses suggested that rapid radiation was the most possible explanation for the lack of resolution. The estimated divergence dates for the clades III, IV, and V (2.524–8.177, 1.94–6.299, and 2.453–7.928 million years ago, respectively) were in a relatively adjacent time period, also supporting the early rapid radiation of the ancestors of *Sinocyclocheius*. These time scales were consistent with the assumption that the ancestors of *Sinocyclocheilus* species were widely distributed in Yungui (Yunnan–Guizhou) Plateau and its southeastern region (Guangxi Zhuang Autonomous Region) in the Tertiary and had diversified to some degree before the end of the Tertiary (Wang and Chen, 2000).

The distribution of *Sinocyclocheilus* species was associated with karst underground waters (Shan et al., 2000). The underground waters in China were mainly distributed in southwestern regions, especially Guangxi, Guizhou, and Yunnan (Yuan et al., 1993). Within *Sinocyclocheilus*, most cave species exhibiting trolomorphic characters are distributed in Guangxi, and most surface species are distributed in Yunnan. The ratio of cave and surface species in Guizhou is intermediate. This distribution pattern of cave species was congruent with the distribution of karst underground waters in these three regions. There were more karst underground rivers in Guangxi than Yunnan and Guizhou (Yuan et al., 1993). However, there were more surface shallow lakes in Yunnan and Guizhou before the uplifting of the Yungui Plateau (Bureau of geology and mineral resources of Guizhou Province, 1987; Bureau of geology and mineral resources of Yunnan Province, 1990). With the gradual uplifting of Guangxi since the Pleistocene (Li, 1984), the ancestral populations of *Sinocyclocheilus* in Guangxi entered different underground rivers. Since the underground rivers were usually small and short (Yuan et al., 1993), gene flows among these ancestral populations were obstructed by geographic isolation, resulting in formation more species exhibiting troglomorphic features.

Interestingly, all species distributed in Yunnan except for two cave species *S. rhinocerous* and *S. hyalinus* clustered as a monophyletic assemblage (clade V) in all our analyses with strong support. This result suggested that Yunnan might be a centre of diversification in *Sinocyclocheilus*. The elevations of distributional geographic localities of *S. rhinocerous* and *S. hyalinus* are lower than those of other species in Yunnan, with the sole exception of *S. angustiporus* because of *S. hyalinus* and *S. angustiporus* in sympatry (Chen et al., 1994). Since *S. angustiporus* is widely distributed and distantly related with *S. hyalinus*, the sympatric phenomenon of this species pair may be suggested that the former dispersed into distribution range of the latter. The elevation of Yunnan is wholly higher than those of Guangxi and Guizhou. The tectonic movements suggested that the uplifting of Yunnn Plateau was closely correlated with the intensive uplifting of Tibet (Qinghai-Xizang) Plateau in the Late Pliocene and Early Pleistocene (Bureau of geology and mineral resources of Yunnan Province, 1990; Huang, 1960). Geological studies demonstrated that the intensive uplifting of Tibet (Qinghai-Xizang) Plateau occurred since 3.4 million years ago (Li et al., 1995). The estimated divergence time for clade V was 2.453–7.928 million years ago and roughly consistent with the uplifting of Yunnan Plateau associated with the intensive uplifting of Tibet Plateau since 3.4 million years ago (Li et al., 1995). Our phylogenetic relationships among species in Yunnan indicated that the diversification of this genus in Yunnan may be correlated with the uplifting of Yunnan Plateau. However, estimates of divergence time based on molecular clock calibration rates should be treated cautiously, given that these calibrations were derived from other species analyses (Zhang and Ryder, 1995).

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