Zootaxa 4476 (1): 130–140
http://www.mapress.com/j/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.4476.1.12
http://zoobank.org/urn:lsid:zoobank.org:pub:B81E10CE-091C-445C-8349-E27FBA7E7AB1

Testing the validity of two putative sympatric species from *Sinocyclocheilus* (Cypriniformes: Cyprinidae) based on mitochondrial cytochrome b sequences

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Abstract

There are over 60 species within the freshwater fish genus *Sinocyclocheilus* (Cypriniformes: Cyprinidae) distributed throughout the Yunnan-Guizhou Plateau and its surrounding areas in China. In recent years, the increasing number of new species described has raised some controversy about the validity of several species within this genus, notably the putative sympatric species pair *S. qiubeiensis* and *S. jiuchengensis*. To test the validity of *S. qiubeiensis* and *S. jiuchengensis*, we analyzed the complete sequences of the mitochondrial cytochrome *b* (CYTB) gene of 20 identified species and one out-group species. Phylogenetic relationships were reconstructed using CYTB with maximum likelihood (ML) and Bayesian inference (BI) methods. Our phylogenetic results showed that all individuals of *S. qiubeiensis* and *S. jiuchengensis* clustered in one clade with strong support. In addition, the genetic distance between the two species was 0.11%, within the range observed at the intraspecific level. The most recent common ancestor of *S. qiubeiensis* and *S. jiuchengensis* dated back to 0.13 million years ago, indicating little time for complete speciation to have occurred. These results clearly support the viewpoint that *S. jiuchengensis* is a synonym of *S. qiubeiensis*.

Key words: Mitochondrial DNA, CYTB, molecular identification

Introduction

The freshwater fish genus *Sinocyclocheilus* (Cypriniformes: Cyprinidae) is endemic to China and is distributed in the Yunnan-Guizhou Plateau (Eastern Yunnan Province and Southern Guizhou Province) and the surrounding Northwestern Guangxi Zhuang Autonomous Region (Zhao & Zhang 2009). The genus *Sinocyclocheilus* harbors a rich species diversity, with a total of 55 species reported by the end of 2009 (Zhao & Zhang 2006; Romero *et al.* 2009). Additionally, there is an increasing number of new species reported in recent years, including *S. huangtianensis* Zhu, Zhu, and Lan 2011, *S. xichouensis* Pan, Li, Yang, and Chen 2013, *S. anshuiensis* Gan, Wu, Wei, and Yang 2013, *S. jinxiensis* Zheng, Xiu, and Yang 2013, *S. gracilicaudatus* Wang, Zhao, Yang, and Zhang 2014, and *S. brevifinus* Li, Li, and Mayden 2014. There are currently over 60 species within the genus *Sinocyclocheilus*, which inhabit different environments (surface and/or cave waters) and display diverse morphological characteristics including varying degrees of eye and pigment degeneration (Romero 2001; Romero & Paulson 2001).

With the increasing number of new species reported, it is becoming more difficult to perform comparative morphological analyses between putative new species and previously known species. This has caused controversies about the validity of some species within this genus. For instance, two putative sympatric species *S. qiubeiensis* Li, Liao, and Yang 2002 and *S. jiuchengensis* Li, Liao, and Yang 2002 from a vauclusian spring were initially identified and described as two distinct species because of differences in body squamation (Li *et al.* 2002).

Accepted by P. Ciccotto: 17 May 2018; published: 13 Sept. 2018

Li *et al*. (2002) observed that the body scales of *S. qiubeiensis* were partially covered and irregularly arranged, whereas those of *S. jiuchengensis* were fully covered and regularly arranged. However, other researchers considered the degree of body squamation to be an unreliable taxonomic characteristic and thus claimed that *S. jiuchengensis* was a synonym of *S. qiubeiensis* (Zhao & Zhang 2009). If these two putative sympatric species are indeed valid species, they could be used as an excellent case for studying sympatric speciation within this genus. Therefore, it is of critical importance to have independent molecular evidence to examine if *S. jiuchengensis* and *S. qiubeiensis* are two valid species.

In recent years, molecular markers have been used as complementary tools for species identification and to settle taxonomic discrepancies within the genus *Sinocyclocheilus*. For example, using sequences of the mitochondrial gene cytochrome b (CYTB), Liang *et al*. (2011) successfully resolved a taxonomic controversy between *S. tianeensis* Li, Xiao, Zan, Luo, Ban, and Fen 2003 and *S. furcodorsalis* Chen, Yang, and Lan 1997, confirming both to be one species under the name *S. tianeensis* (Liang *et al.* 2011). In addition, Li *et al*. (2008) used sequences of CYTB and NADH dehydrogenase subunit 4 (ND4) to examine whether *S. lunnanensis* Li 1985 and *S. halfibindus* Li and Lan 1992 were synonyms of *S. oxycephalus* Li 1985 and *S. microphthalmus* Li 1989, respectively. Collectively, it has been established that the CYTB gene is an efficient and valid maker for species delineation (Johns & Avise 1998), as also demonstrated in examples of *Sinocyclocheilus* (Li *et al.* 2008; Liang *et al.* 2011). It is therefore reasonable to believe that CYTB could be informative for species delineation of two putative sympatric species: *S. qiubeiensis* and *S. jiuchengensis*.

To examine the validity of the two putative sympatric species *S. qiubeiensis* and *S. jiuchengensis*, we conducted phylogenetic analyses of species of *Sinocyclocheilus* distributed in Southeastern Yunnan based on CYTB sequences. Southeastern Yunnan is characterized by the typical karst landforms with complex and intersecting underground water bodies, and harbors approximately 30 species of *Sinocyclocheilus* species, including *S. qiubeiensis* and *S. jiuchengensis*.

Materials and methods

Samples. A total of 40 individuals of three putative species (*S. qiubeiensis, S. jiuchengensis,* and *S. maculatus*) were collected from Yanshan County and Qiubei County of Yunnan Province, China (Table 1). Tissue samples were preserved in 100% ethanol and voucher specimens were deposited in the Zoological Museum of Yunnan University. In addition, two samples of *S. qiubeiensis* (collected from Songming County) reported by Li *et al.* (2008) were analyzed in this study. All previously published CYTB sequences of the species of *Sinocyclocheilus* distributed in the Yunnan Province were retrieved from GenBank for phylogenetic analyses (Table 2). Based on phylogenetic relationships among cyprinid species (Wang *et al.* 2007), *Barbodes laticeps* was chosen as the outgroup for phylogenetic analyses in this study and an additional seven outgroup species were used for estimating the divergence times (Table 2).

DNA preparation, PCR amplification and sequencing. Genomic DNA was extracted from muscle tissues using a TIANamp Genomic DNA Kit (TianGen Biotech Co. Ltd, Beijing) according to the manufacturer's instructions. The complete CYTB gene sequences were amplified using polymerase chain reactions (PCR) with two primers: SCytb03F CTCAGACTTTAACTGAGACC and SCytb04R GAATTCTGGCTTTGGGA (Liang *et al.* 2011). PCR amplifications were carried out in 50 µl reaction mixtures consisting of 5 µl 10 × PCR buffer, 0.125 mM $MgCl_2$, 0.01 mM dNTPs, 0.1 µM each primer, 1.2 U Taq DNA polymerase (Takara Biotechnology Co. Ltd, Dalian), and approximately 50 ng of genomic DNA. Amplification was carried out with denaturing at 94°C for 5 min, 35 cycles of denaturing at 94°C for 60 s, annealing at 46.2°C for 60 s, and extension at 72°C for 60 s, followed by extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gel and successful amplifications were sent to BGI-Shenzhen for Sanger-sequencing.

Data analyses. Raw sequence traces were checked and aligned using the software package DNASTAR v7.1 (DNASTAR Inc., Madison, WI, USA). All sequences have been deposited in the GenBank database with accession nos. MF324971–MF325010 (Table 2). The CYTB gene sequences were aligned in ClustalW implemented in MEGA6 (Tamura *et al.* 2013). The aligned sequence data was later used to calculate nucleotide compositions, pairwise distances, and transition/transversion ratios in MEGA6 (Tamura *et al.* 2013). Nucleotide substitution saturation was analyzed in DAMBE (Xia & Xie 2001). Diversity measures and the number of haplotypes were calculated in DnaSP5.10 (Librado & Rozas 2009). Median-Joining networks among haplotype sequences were constructed by POPART (Leigh & Bryant 2015). Neutrality tests Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu & Li 1993) for the datasets of *S. qiubeiensis*_P1 (samples from Jiulongtan of Quibei County), *S. qiubeiensis*_P2 (samples from Baima of Quibei County), and *S. qiubeiensis*_P3 (samples from Dayile of Quibei County) were calculated using DnaSP5.10 and Arlequin3.0 (Excoffier *et al.* 2005; Librado & Rozas 2009).

Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) analyses. The optimal nucleotide substitution model was selected using likelihood ratio tests in jModeltest 2.17 (Posada 2008). The optimal model was GTR+G. The ML tree was constructed in PAUP* 4.0b10 (Swofford 2003) using a heuristic search and tree-bisection-reconnection (TBR) branch swapping. Confidence values on nodes were assessed using non-parametric bootstrapping (Felsenstein 1985) with 1000 replications. It is usually considered that a clade is supported if the bootstrap percentage is equal or greater than 70% (Hillis & Bull 1993).

We performed Bayesian analyses with MrBayes v3.2 (Ronquist & Huelsenbeck 2003). The best-fitting nucleotide substitution model GTR+G was applied. Random starting trees were used and analyses were run for 1×10^7 generations using four independent chains (one cold and three heated), sampling the Markov chain at intervals of 100 generations for a total of 1×10^5 generations. The samples prior to reaching stationary (25,000 trees) were discarded as burn-in and the remaining trees were then used to generate a majority-rule consensus tree. It is generally considered that a clade is strongly supported if the posterior probability is equal or greater than 95% (Leaché & Reeder 2002).

The divergence times were estimated using the MCMCtree program of PAML (Yang 2007) using the Bayesian relaxed clock method. To achieve computational efficiency, a normal approximation to the maximum likelihood estimates (MLEs) of branch lengths was used. The substitution rate was estimated by the BASEML program of PAML4.4 (Yang 2007). The shape parameter (α) and the scale parameter (β) for the gamma prior of the root rate parameter and the rate drift parameters were determined by suggested procedures. The denoted nodes C1 and C2 were used as calibration points. C1 was based on geologic evidence (0.15 Ma, set as a minimum) of the separation of Qinghai Lake from the Upper Yellow River (Li *et al.* 2001). C2 was based on molecular evidence (15.96 Ma, set as a maximum) for the divergence of Cyprinini and Labeonini (Wang *et al.* 2007). The first 1×10^6 generations were discarded as burn-in. The subsequent 1.0×10^8 generations were included in the analyses. The sample frequency was per 1000 generations.

Results

Sequence characteristics and haplotype analyses. We obtained the complete CYTB (1140 bp) sequences of 40 individuals. None of these protein-coding sequences had premature stop codons or ambiguous nucleotides in translation, indicating that these sequences were functional genes (Zardoya & Meyer 1996). The mean nucleotide base compositions were A 29.1%, C 28.4%, G 15.2%, and T 27.3% across all sites for all sequences. The transition/transversion ratio (R) was 5.89. These characteristics are typical for mitochondrial protein-coding genes (Cantatore *et al.* 1994). In addition, there were 107 variable sites defining 7 haplotypes (Hap1–7) identified among all sequences of *S. qiubeiensis* and *S. jiuchengensis*, including two reported sequences of *S. qiubeiensis* reported by Li *et al*. (2008) (Table 1 and Fig. 1). All samples of *S. qiubeiensis* and *S. jiuchengensis* collected from the type locality (Qiubei County) shared the main haplotype expect for one sample of *S. qiubeiensis* from Dayile of Quibei County. However, two sequences of *S. qiubeiensis* (*S. qiubeiensis*_D) reported by Li *et al*. (2008) did not cluster with our samples. Furthermore, the genetic distance between our sequences of *S. qiubeiensis* and the two sequences of *S. qiubeiensis* reported by Li *et al*. (2008) is 9.41%, much higher than the threshold of 2% for intraspecific divergence (Johns & Avise 1998).

Phylogenetic analyses. The Bayesian tree is shown in Fig. 2. The topology of the ML tree (not shown) was essentially identical to that of the Bayesian tree. On the Bayesian tree, three major clades (I, II, and III) could be identified. However, the exact phylogenetic positions among these three clades were not resolved (Fig. 2). Noticeably, the two putative sympatric species *S. qiubeiensis* and *S. jiuchengensis* within the clade III grouped together as a monophyletic subclade with strong support (BPP = 1). Given that *S. qiubeiensis* and *S. jiuchengensis* are distributed in Qiubei County of Yunnan Province, the phylogenetic pattern among *S. qiubeiensis*_P1, *S. jiuchengensis*, *S. qiubeiensis*_P2, and *S. qiubeiensis*_P3 indicates these samples likely belong to one single species.

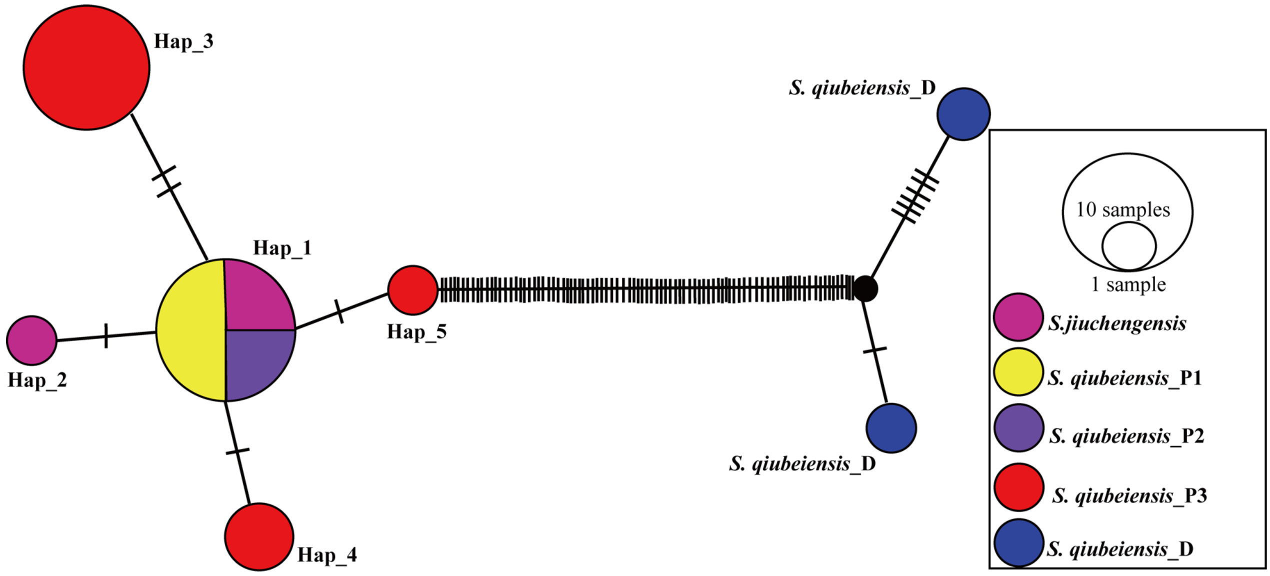

FIGURE 1. The Median-joining network for 7 CYTB haplotypes of all sampled *S. qiubeiensis* and *S. jiuchengensis*.

Genetic distance analyses. The level of interspecific genetic divergence is the main criterion for species identification by molecular methods (Peng *et al.* 2009). Previous studies demonstrated that interspecific divergence was equal to or larger than 2% for CYTB (Johns & Avise 1998). The CYTB genetic distance between *S. qiubeiensis* (collected from Qiubei County) and *S. jiuchengensis* was 0.11%, while intraspecific genetic distances of *S. qiubeiensis* and *S. jiuchengensis* were 0.14% and 0.09%, respectively (Supplementary Table S1). All values were less than the threshold of 2% for interspecific divergence of CYTB gene among animal species. This indicated that genetic divergence between *S. qiubeiensis* (collected from Qiubei County) and *S. jiuchengensis* was at the levels typically observed within a species, further supporting them as one single species.

The most recent common ancestor estimation and neutrality tests. Our results showed that the most recent common ancestor (MRCA) of the sampled species of *Sinocyclocheilus* originated about 11 million years ago (Mya) and the MRCA of *S. qiubeiensis* (collected from Qiubei County) and *S. jiuchengensis* dated back to about 0.13 Mya (Fig. 3).

All samples of *S. qiubeiensis* collected from Qiubei County were treated as one population for conducting neutrality tests. The values for Tajima's *D* and Fu's *Fs* were 0.7064 ($P > 0.1$) and 0.32652 ($P > 0.1$), respectively. Results of both tests indicated no signs of population expansion and suggested long-term population stability.

Discussion

The validity of *S. qiubeiensis* and *S. jiuchengensis*. The results support the viewpoint that *S. jiuchengensis* and *S. qiubeiensis* (collected from Quibei County) are not two valid species and the former is a synonym of the latter (Zhao & Zhang 2009). In this study, three lines of molecular evidence support this viewpoint. First, *S. jiuchengensis* and *S. qiubeiensis* (collected from Quibei County) shared the same haplotype (Hap_1) in the median-joining network (see Fig. 1). Second, on the phylogenetic tree, the CYTB gene sequences from two putative species intermingled and formed a monophyletic subclade (rather than two distinct clades) with strong support (see Fig. 2). For two different valid species, it is impossible to share the same haplotype for the relatively fast-evolving CYTB gene, assuming no introgression occurred. Third, the level of genetic divergence (0.11%) between *S. jiuchengensis* and *S. qiubeiensis* did not reach the interspecific level. A widely accepted routine demonstrated that interspecific genetic divergence at the CYTB gene between closely related vertebrate species (including fishes) was generally greater than 2%, while intraspecific genetic divergence at this gene was generally less than 1% (rarely greater than 2%) (Johns & Avise 1998). According to this threshold, genetic divergence between the two putative species *S. jiuchengensis* and *S. qiubeiensis* was still at the intraspecific level. Finally, the time to the MRCA of the two putative species was 0.13 Mya, a short time that was likely insufficient for complete speciation to have occurred.

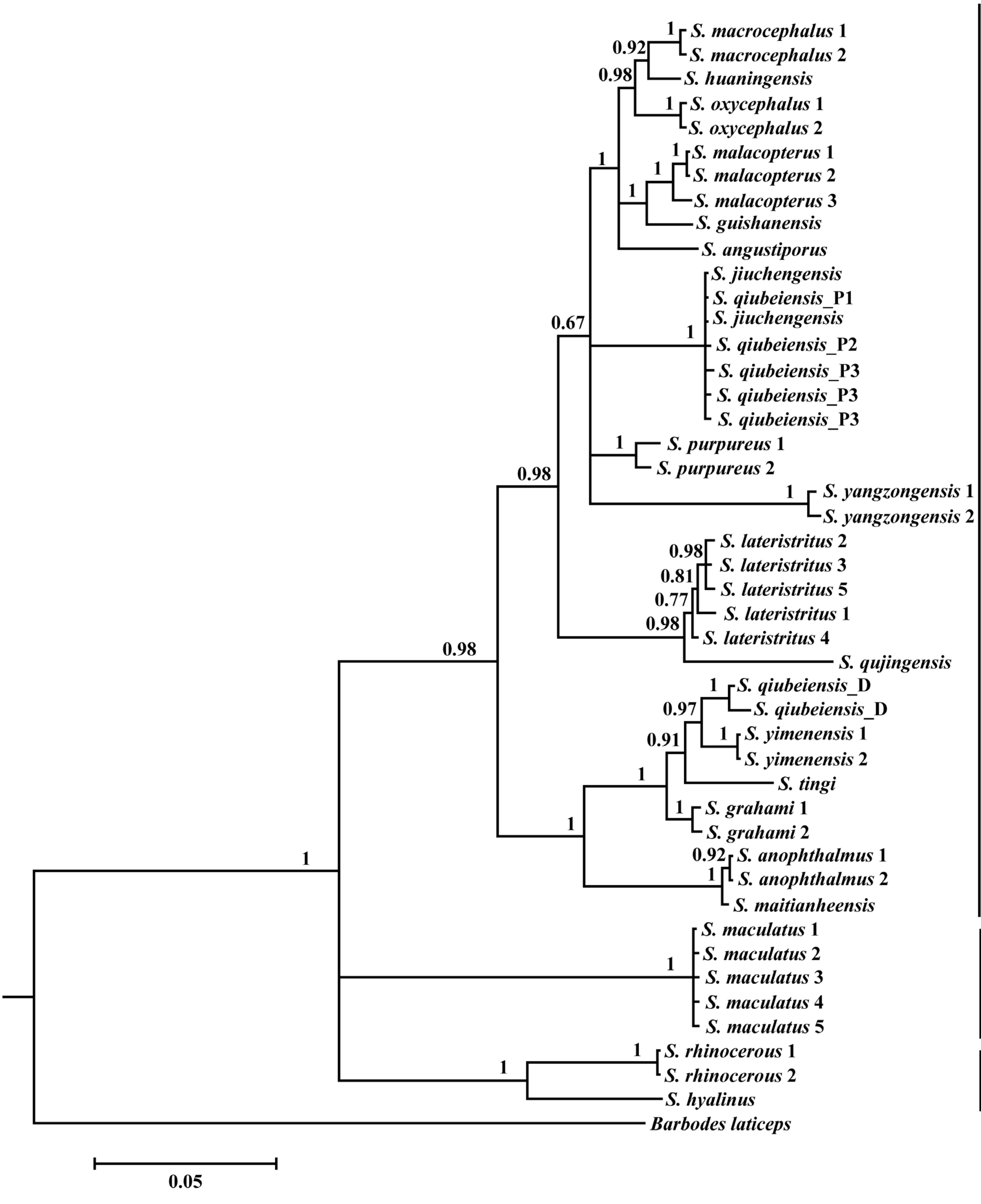

FIGURE 2. The Bayesian tree constructed based on CYTB sequences of species of *Sinocyclocheilus* from Yunnan. Node support values are Bayesian posterior probabilities.

The main morphological difference between *S. jiuchengensis* and *S. qiubeiensis* is body squamation (Li *et al.* 2002) (Supplementary Figure S1). However, this characteristic has been considered unreliable for species taxonomy and cladistic analyses. This was supported by two previous morphological studies that generated contradictory phylogenetic results because of different weights given to scales and other characteristics (Shan & Yue 1994; Wang *et al.* 1999). When mapping the scale characteristics on a molecular phylogeny of *Sinocyclocheilus*, it was clearly indicated that this trait is phylogenetically uninformative (Xiao *et al.* 2005).

Collectively, our independent molecular evidence further supported that *S. jiuchengensis* is a synonym of *S. qiubeiensis* (Zhao & Zhang 2009).

TABLE 1. List of species, localities, and haplotype information for species from southeastern Yunnan. *N* indicates the number of samples. P1, P2, and P3 indicate samples collected from Jiulongtan, Baima and Dayile of Qiubei County, respectively. D indicates samples/sequences downloaded from the NCBI GenBank database. The numbers in parentheses indicates the number of sequences.

Species	Locality	N	Haplotype
*S. qiubeiensis*_P1	Jiulongtan, Qiubei County, Yunnan	6	Hap_1 (6)
*S. qiubeiensis*_P2	Baima, Qiubei County, Yunnan	3	Hap_1 (3)
*S. qiubeiensis*_P3	Dayile, Qiubei County, Yunnan	15	Hap_3 (11), 4 (3), Hap_5 (1)
S. jiuchengensis	Jiulongtan, Qiubei County, Yunnan	4	Hap_1 (3), 2 (1)
*S. qiubeiensis*_D	Songming County, Yunnan, Li *et al.* (2008).	2	Hap_6 (1),7 (1)
S. maculatus	Beishapo, Yanshan County, Yunnan	12	Hap_8 (5),9 (1), 10 (1), 11 (1), 12 (4)

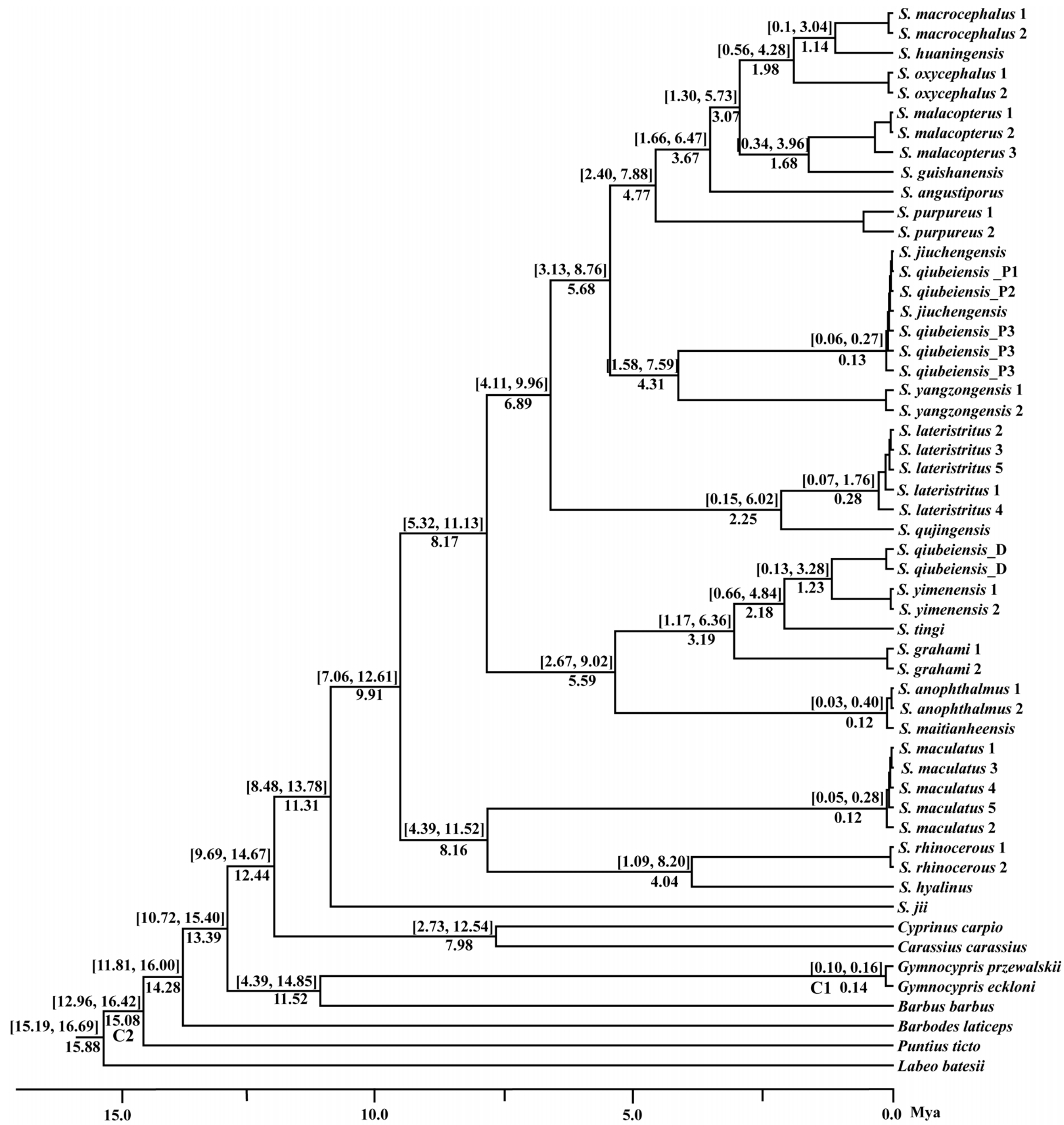

FIGURE 3. Chronogram of the *Sinocyclocheilus* and outgroup species based on Bayesian relaxed clock analyses. Time scale is in millions of years ago (Mya). C1 and C2 denote the nodes used for calibrating molecular date estimates. The numbers above the nodes indicate the estimated divergence times, while the numbers below the nodes indicate the 95% credible interval on time estimates.

TABLE 2. List of species, localities, and GenBank accession numbers for species previously studied.

Species	Locality	Accession No.
S. hyalinus	Alugudong, Luxi County, Yunnan	AY854721
S. tingi	Fuxianhu Lake, Yunnan	AY854701
S. guishanensis	Guishan, Shilin County, Yunnan	AY854722
S. oxycephalus	Heilongtan,Shilin County,Yunnan	Y854685, AY854686
S. macrocephalus	Heilongtan,Shilin County,Yunnan	AY854684, AY854683
S. huaningensis	Huaning County, Yunnan	AY854718
S. anophthalmus	Jiuxiang,Yiliang County,Yunnan	NC_023472, KF892542
S. maitianheensis	Jiuxiang,Yiliang County, Yunnan	AY854710
S. lateristritus	Luliang County, Yunnan	AY854703–AY854707,
S. malacopterus	Luoping County, Yunnan	AY854697–AY854699
S. purpureus	Luoping County, Yunnan	U366189, EU366194
S. rhinocerous	Luoping County, Yunnan	AY854720, NC_027168
S. angustiporus	Luxi County, Yunnan	AY854702
S. grahami	Qinglongsi, Kunming, Yunnan	AY854696, AY854694
*S. qiubeiensis*_D	Songming County, Yunnan	EU366195, EU366188
S. qujingensis	Wujiafen, Qujing County, Yunnan	AY854719
S. yangzongensis	Yangzonghai Lake, Yunnan	AY854725, AY854726
S. yimenensis	Yimen County, Yunnan	EU366191, EU366192
*S. qiubeiensis*_P1	Jiulongtan, Qiubei County, Yunnan	MF324978–MF324983
*S. qiubeiensis*_P2	Baima, Qiubei County, Yunnan	MF324971–MF324973
*S. qiubeiensis*_P3	Dayile, Qiubei County, Yunnan	MF324984–MF324998
S. jiuchengensis	Jiulongtan, Qiubei County, Yunnan	MF324974–MF324977
S. maculatus	Beishapo, Yanshan County, Yunnan	MF324999–MF325010
S. jii		AY854727
Barbodes laticeps		AY854738
Cyprinus carpio		NC_001606
Carassius carassius		NC_006291
Barbus barbus		NC_008654
Puntius ticto		NC_008658
Labeo batesii		AB238967
Gymnocypris przewalskii		AB239595
Gymnocypris eckloni		AY463522

Misidentification of *S. qiubeiensis* samples in one previous study. Li *et al.* (2008) initially reported two CYTB sequences of *S. qiubeiensis* (collected from Songming Country, Yunnan) in a phylogenetic and divergence time estimation study. If the two CYTB sequences belonged to *S. qiubeiensis*, this would imply that geographic distance (approximately 185 km between Songming and Qiubei) did not play a substantial role in shaping genetic divergence within *S. qiubeiensis*. Unexpectedly, our phylogenetic analyses showed that two CYTB sequences from *S. qiubeiensis* from Songmin did not cluster with our sequences of *S. qiubeiensis* from Qiubei as one monophyletic clade (Fig. 2). The genetic distance between sequences of *S. qiubeiensis* from Songming and Qiubei was 9.41% (Supplementary Table S1), much higher than the threshold of 2% for intraspecific divergence (Johns & Avise 1998). Given that our samples were collected from the type locality (Quibei County), *S. qiubeiensis* from Songming reported by Li *et al.* (2008) is likely another species. This suggests that one must collect samples from the type locality or alternatively ensure that the species is correctly identified for molecular phylogenetic and evolutionary biology studies on *Sinocyclocheilus*.

Acknowledgements

This work was partially supported by the National Nature Science Foundation of China (31560111) and the Graduate Student Scientific Research Innovation Project in Yunnan University. S.C. was supported by the Hundred Oversea Talents Recruitment Program of Yunnan Province.

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SUPPLEMENTARY TABLE S1. The genetic distances among CYTB sequences of species of *Sinocyclocheilus* under the Kimura-2-parameter model.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Intraspecific genetic distance
1	***S. jiuchengensis***																							0.001
2	***S. qiubeiensis* (this study)**	0.001																						0.001
3	***S. maculatus***	0.150	0.149																					
4	***S. angustiporus***	0.056	0.056	0.143																				-
5	***S. guishanensis***	0.058	0.058	0.149	0.040																			-
6	***S. huaningensis***	0.049	0.049	0.143	0.039	0.030																		-
7	***S. lateristritus***	0.069	0.069	0.138	0.066	0.064	0.060																	0.005
8	***S. macrocephalus***	0.053	0.053	0.142	0.034	0.028	0.018	0.059																0.002
9	***S. malacopterus***	0.051	0.051	0.143	0.037	0.023	0.033	0.057	0.031															0.005
10	***S. oxycephalus***	0.046	0.046	0.144	0.034	0.033	0.022	0.064	0.025	0.036														0.002
11	***S. purpureus***	0.052	0.053	0.139	0.040	0.042	0.035	0.060	0.035	0.037	0.042													0.010
12	***S. yangzongensis***	0.082	0.083	0.155	0.080	0.083	0.083	0.095	0.079	0.075	0.081	0.076												0.004
13	***S. grahami***	0.089	0.089	0.142	0.083	0.088	0.083	0.087	0.086	0.081	0.084	0.081	0.111											0.004
14	***S. yimenensis***	0.090	0.090	0.145	0.088	0.091	0.093	0.094	0.091	0.083	0.092	0.085	0.106	0.026										0.000
15	***S. qiubeiensis* (Li et al. 2008)**	0.094	0.094	0.147	0.087	0.095	0.094	0.094	0.093	0.087	0.091	0.086	0.113	0.026	0.020									0.006
16	***S. qujingensis***	0.099	0.099	0.164	0.098	0.091	0.093	0.043	0.093	0.088	0.095	0.093	0.116	0.114	0.125	0.123								-
17	***S. maitianheensis***	0.100	0.100	0.147	0.091	0.093	0.089	0.094	0.091	0.087	0.092	0.086	0.100	0.062	0.069	0.072	0.119							-
18	***S. anophthalmus***	0.101	0.101	0.148	0.095	0.096	0.093	0.095	0.095	0.090	0.096	0.087	0.099	0.063	0.070	0.073	0.121	0.003						0.000
19	***S. tingi***	0.103	0.103	0.149	0.092	0.104	0.101	0.099	0.103	0.095	0.098	0.098	0.116	0.036	0.038	0.039	0.130	0.077	0.078					-
20	***S. hyalinus***	0.132	0.131	0.141	0.130	0.126	0.127	0.125	0.126	0.123	0.129	0.128	0.135	0.138	0.137	0.138	0.143	0.142	0.140	0.151				-
21	***S. rhinocerous***	0.132	0.131	0.134	0.134	0.129	0.131	0.121	0.128	0.126	0.131	0.130	0.150	0.136	0.141	0.141	0.138	0.142	0.143	0.151	0.065			0.000
22	***Barbodes laticeps***	0.173	0.172	0.164	0.163	0.161	0.164	0.166	0.161	0.161	0.166	0.167	0.174	0.162	0.167	0.169	0.146	0.167	0.168	0.175	0.157	0.159	0.000	-

SUPPLEMENTARY FIGURE S1. Comparison of the body squamation between two putative species of *Sinocyclocheilus*. *Sinocyclocheilus qiubeiensis* (left) possesses tiny scales irregularly arranged and incompletely covering the body, with most of the scales concentrated on the dorsal and ventral sides of the lateral line. *Sinocyclocheilus jiuchengensis* (right) possesses dense scales regularly arranged and completely covering the body.