Molecular phylogenetic evidence that the Chinese viviparid genus *Margarya* (Gastropoda: Viviparidae) is polyphyletic

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We investigated the phylogeny of the viviparid genus *Margarya*, endemic to Yunnan, China, using two mitochondrial gene fragments (COI and 16S rRNA). The molecular phylogeny based on the combined dataset indicates that *Margarya* is polyphyletic, as two of the three well-supported clades containing species of *Margarya* also comprise species from other viviparid genera. In one clade, sequences of four species of *Margarya* even cluster indiscriminately with those of two species of *Cipangopaludina*, indicating that the current state of Asian viviparid taxonomy needs to be revised. Additionally, these data suggest that shell evolution in viviparids is complex, as even the large and strongly sculptured shells of *Margarya*, which are outstanding among Asian viviparids, can apparently be easily converted to simple smooth shells. The molecular data also indicate that the species status of the six extant species of *Margarya* should be re-assessed.

***Margarya*, molecular phylogeny, polyphyly, mtDNA China**

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The ten freshwater lakes of Yunnan, in the southwest plateau of China, host many endemic macrophytes and animals including fishes, mollusks and crustaceans [1–9]. The lakes, of which Er-hai, Dianchi, and Fuxian are the largest (Figure 1), were formed during the late Pliocene and early Pleistocene [10]. Today, they drain into three different river systems (compare Figure 1) [10,11].

The viviparid *Margarya* Nevill, 1877 is endemic of the Yunnan lakes. First discovered by the Margary expedition to Ta-li fu (modern day Lake Er-hai), Nevill (1877) set up the genus based on the type species *Margarya melanioides* collected from that lake [12,13]. *Margarya* has been described solely based on shell characters, viz. its large scalariform shells with a distinct suture and strong spiral sculpture

(Nevill, 1877; Figure 2) which make it easily distinguishable from all other Asian viviparids [12]. While other viviparid genera found in Yunnan, notably *Cipangopaludina* Hannibal, 1912 and *Sinotaia* Haas, 1939, differ in that they are unsculptured and smaller (*Sinotaia*), a great degree of shell variability is also found within *Margarya* (Figure 2) [14,15]. Considerable variation in sculpture has also been observed in the type species just within Lake Dianchi (Figure 2) [16].

Till now, a total of ten *Margarya* species, including three fossil taxa and a recently extinct species, have been described [12,14,17–20]. The six extant species in the genus *Margarya* occur in eight lakes, viz. Er-hai, Dianchi, Yangzonghai, Fuxian, Xingyun, Qilu lakes and Lake Yilong and Datun are [15,19]. With the exception of one species (*Margarya yangtsunghaiensis*), all extant species of *Margarya*

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Figure 1 Map of sampling localities of each species in Yunnan Province, southwestern China. ★, *M. melanioides*; ▲, *M. yangtsunghaiensis*; ▼, *M. mansuyi*; ●, *M. bicotata*; ■, *M. monodi*; ○, *M. tropidophora*.

Figure 2 The shells of *Margarya*. A, *Margarya melanioides* (IOZ-FG164208), Lake Er-hai. B, Four types of *Margarya melanioides*. a, A type (KIZ-DA2005002); b, B type (KIZ-DLN2008002); c, C type (KIZ-45); d, D type (KIZ-DLN2008080), Lake Dianchi. C, *Margarya monodi* (KIZ-13), Lake Dianchi. D, *Margarya tropidophora* (IOZ-FG89708), Lake Er-hai. E, *M. yangtsunghaiensis* (holotype IOZ-00001), Lake Yangzonghai. F, *Margarya mansuyi* (KIZ-DLN2007008), Lake Xingyun. G, *Margarya bicotata* (holotype, IOZ), Lake Fuxian. H, *Margarya francheti* (IOZ-FG89664), Lake Dianchi. I, *Margarya elongata* (holotype, IOZ-FG00074), Lake Dianchi. J, *Margarya yini* (holotype, IOZ), Lake Dianchi. K, *Margarya tchangsi* (paratype, IOZ-FG89703), Lake Dianchi. Scale bar=1 cm.

occur in two or more of these lakes [21].

The phylogenetic relationships of *Margarya* to other Asian viviparids entirely unclear. The genus has been described based on shell morphology only and anatomical data are still lacking. The intrageneric systematics of *Margarya* has also been poorly investigated. Based on shell characters, Tchang and Tsi [12] considered *M. yangtsunghaiensis* as the most “primitive” species in the genus *Margarya*. However, Chen and Zhang [22] inferred *M. melanioides* to be the most basal taxon based on karyotypes. Based on a Neighbor-Joining Analysis of *COI* sequences from a single individual of each species, Huang and Wang [23] reconstructed the phylogeny of *Margarya* in Dianchi Lake and suggested that *Margarya* in Lake Dianchi is monophyletic. However, this result is rather inconclusive because of the insufficiencies in sampling and taxon coverage.

In the present study, the phylogenetic relationships of *Margarya* are investigated using the mitochondrial *COI* and 16S rRNA genes based on an extensive sampling of *Margarya*. The primary aim of this study is to test the monophyly of *Margarya* and to uncover its phylogenetic relationship to other viviparids. We also aim to investigate the relationships of *Margarya* species and populations from different lakes and to explore the possibility of within-lake radiation in Lake Dianchi, which hosts four species of *Margarya*.

1 Materials and methods

1.1 Taxon sampling

The specimens of *Margarya* examined in this study were collected primarily between 2005 and 2008. All voucher specimens used in this study were deposited at the Kunming Institute of Zoology, Chinese Academy of Sciences. The

identification of *Margarya* species followed the criteria of Tchang and Tsi [12]. While *Margarya* has been recorded from eight lakes, fresh samples could only be obtained from five lakes (Figure 1). Due to heavy pollution and over harvesting, *M. mansuyi* has disappeared from Lakes Yilong and Datun, and *M. melanioides* has disappeared from Lake Er-hai, but a few specimens of *M. melanioides* were collected from Haixihai reservoir, which forms part of Lake Er-hai. Additionally, we checked all specimens of *Margarya* preserved in the Invertebrate Museum of the Institute of Zoology, Beijing.

A total of 55 specimens have been sequenced, including 42 specimens of all 6 extant species of *Margarya* and 13 specimens of *Cipangopaludina* Hannibal, 1912 from the Yunnan lakes (Table 1). Sequences of other viviparid genera (Table 1) were retrieved from GenBank. Sequences of two species of the ampullariid, *Pomacea bridgesii* (Reeve, 1856) and *P. canaliculata* (Lamarck, 1819), and two species of Viviparinae, *Viviparus ater* (De Cristofori & Jan, 1832) and *V. contectus* (Millet, 1813) from GenBank were used as outgroup to root the phylogeny [24].

1.2 DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from muscle tissue fixed in 99% ethanol. Tissue samples were digested using proteinase K and subsequently purified following a standard phenol/chloroform isolation and ethanol precipitation. Two mitochondrial gene fragments (*COI* and 16S rRNA) were amplified by polymerase chain reaction (PCR) using the primer pairs LCO1490/HCO2198 (*COI*) [25] and 16Sar-L/16Sbr-H (16S) [26]. PCR amplifications were conducted in a total volume of 50 µL using the following cycling conditions: an initial denaturing step at 94°C for 10 min, followed by 35 cycles at 94°C (1 min), annealing at 46–50°C (*COI*: 46°C

Table 1 Species used and GenBank Accession numbers

Taxon	Voucher number	Locality	Country	COI	16S
Outgroup					
<i>Pomacea bridgesii</i>	–	GenBank	S. America	–	EU274500
<i>P. canaliculata</i>	–	GenBank	S. America	EU2744574	EU2744501
<i>Viviparus ater</i>	–	Goldbach	Switzerland	FJ405882	FJ405734
<i>V. contectus</i>	–	Hellebaek lakes	Denmark	FJ405835	FJ405692
Ingroup					
<i>Bellamya jeffreysi</i>	–	Cape Mclear	Malawi	FJ405873	FJ405719
<i>B. jeffreysi</i>	–	River Songwe	Tanzania	FJ405857	FJ405702
<i>B. crawshayi</i>	–	Mkolo	Zambia	FJ405867	FJ405710
<i>Neothauma tanganyicense</i>	–	Mpungu	Zambia	FJ405843	FJ405709
<i>Angulyagra</i> sp.	–	Nam Dinh	Vietnam	FJ405879	FJ405723
<i>Larina</i> sp.	–	Queensland	Australia	–	FJ405733
<i>Mekongia</i> sp.	–	Hanoi	Vietnam	FJ405875	FJ405722
<i>Sinotaia</i> sp.	–	Hanoi	Vietnam	FJ405883	FJ405728
<i>Taia polyzonata</i>	–	Lower pierce reservoir	Singapore	–	FJ405727
<i>Cipangopaludina chinensis</i>	–	GenBank	–	FJ710298	FJ710214

(To be continued on the next page)

(Continued)

Taxon	Voucher number	Locality	Country	COI	16S
<i>C. chinensis</i>	–	GenBank	–	EU528474	FJ710213
<i>C. dianchiensis</i>	D43	Dianchi lake	China	GU198781	GU198835
<i>C. dianchiensis</i>	D44	Dianchi lake	China	GU198782	GU198836
<i>C. dianchiensis</i>	D178	Dianchi lake	China	GU198818	GU198872
<i>C. dianchiensis</i>	D177	Dianchi lake	China	GU198817	GU198871
<i>C. longispria</i>	D148	Yilong lake	China	GU198809	GU198863
<i>C. longispria</i>	D149	Yilong lake	China	GU198810	GU198864
<i>C. sp.</i>	D59	Dianchi lake	China	GU198783	GU198837
<i>C. sp.</i>	D60	Dianchi lake	China	GU198784	GU198838
<i>C. sp.</i>	D61	Dianchi lake	China	GU198785	GU198839
<i>C. sp.</i>	D62	Dianchi lake	China	GU198786	GU198840
<i>C. sp.</i>	D63	Dianchi lake	China	GU198787	GU198841
<i>C. sp.</i>	D65	Dianchi lake	China	GU198788	GU198842
<i>C. sp.</i>	D66	Dianchi lake	China	GU198789	GU198843
<i>C. sp.</i>	D67	Dianchi lake	China	GU198790	GU198844
<i>C. sp.</i>	D68	Dianchi lake	China	GU198791	GU198845
<i>C. sp.</i>	D179	Dianchi lake	China	GU198819	GU198873
<i>Margarya melanioides</i> A	D8	Dianchi lake	China	GU198766	GU198821
<i>M. melanioides</i> A	D17	Haixihai lake	China	GU198767	GU198822
<i>M. melanioides</i> A	D79	Haixihai lake	China	GU198792	GU198846
<i>M. melanioides</i> A	D80	Haixihai lake	China	GU198793	GU198847
<i>M. melanioides</i> A	D81	Haixihai lake	China	GU198794	GU198848
<i>M. melanioides</i> B	D86	Dianchi lake	China	GU198795	GU198849
<i>M. melanioides</i> B	D129	Dianchi lake	China	GU198800	GU198854
<i>M. melanioides</i> B	D134	Dianchi lake	China	GU198801	GU198855
<i>M. melanioides</i> B	D135	Dianchi lake	China	GU198802	GU198856
<i>M. melanioides</i> C	D2	Dianchi lake	China	GU198765	GU198820
<i>M. melanioides</i> C	D122	Dianchi lake	China	GU198797	GU198851
<i>M. melanioides</i> C	D123	Dianchi lake	China	GU198798	GU198852
<i>M. melanioides</i> C	D127	Dianchi lake	China	GU198799	GU198853
<i>M. melanioides</i> D	D136	Dianchi lake	China	GU198803	GU198857
<i>M. mealnioides</i> D	D141	Dianchi lake	China	GU198808	GU198862
<i>M. bicotata</i>	D39	Fuxian lake	China	GU198780	–
<i>M. bicotata</i>	D172	Dianchi lake	China	GU198812	GU198866
<i>M. bicotata</i>	D173	Dianchi lake	China	GU198813	GU198867
<i>M. bicotata</i>	D174	Dianchi lake	China	GU198814	GU198868
<i>M. bicotata</i>	D175	Dianchi lake	China	GU198815	GU198869
<i>M. bicotata</i>	D176	Dianchi lake	China	GU198816	GU198870
<i>M. mansuyi</i>	D21	Xingyun lake	China	GU198768	GU198823
<i>M. mansuyi</i>	D22	Xingyun lake	China	GU198769	GU198824
<i>M. mansuyi</i>	D24	Xingyun lake	China	GU198770	GU198825
<i>M. mansuyi</i>	D25	Xingyun lake	China	GU198771	GU198826
<i>M. mansuyi</i>	D26	Qilu lake	China	GU198772	GU198827
<i>M. mansuyi</i>	D27	Qilu lake	China	GU198773	GU198828
<i>M. mansuyi</i>	D28	Qilu lake	China	GU198774	GU198829
<i>M. mansuyi</i>	D29	Qilu lake	China	GU198775	GU198830
<i>M. mansuyi</i>	D30	Qilu lake	China	GU198776	GU198831
<i>M. monodi</i>	D87	Dianchi lake	China	GU198796	GU198850
<i>M. monodi</i>	D137	Dianchi lake	China	GU198804	GU198858
<i>M. monodi</i>	D138	Dianchi lake	China	GU198805	GU198859
<i>M. monodi</i>	D139	Dianchi lake	China	GU198806	GU198860
<i>M. monodi</i>	D140	Dianchi lake	China	GU198807	GU198861
<i>M. tropidophora</i>	D171	Dianchi lake	China	GU198811	GU198865
<i>M. yangtsunghaiensis</i>	D33	Yangzonghai lake	China	GU198777	GU198832
<i>M. yangtsunghaiensis</i>	D35	Yangzonghai lake	China	GU198778	GU198833
<i>M. yangtsunghaiensis</i>	D36	Yangzonghai lake	China	GU198779	GU198834

for 3 min 30 s; 16S rRNA: 50°C for 1 min) and extension at 72°C (1 min 45 s for COI; 1 min for 16S rRNA) with a final extension step of 10 min at 72°C. PCR products were purified via spin columns. Sequencing was performed directly using the corresponding PCR primers. DNA sequences of both strands were obtained using the BigDye Terminator v3.1 on an ABI PRISM 3730 following the manufacturer's instructions. All new sequences have been deposited in GenBank (Accession Nos: GU198765–GU198873).

1.3 Phylogenetic analyses

Sequences were aligned using Clustal X v1.83 with default gap costs and were revised by eye to maximize the homology of positions [27]. Sequences of 1148 bp were unambiguously aligned for all taxa (641 bp of COI and 507 bp of 16S). To explore the saturation of the sequences, transitions and transversions were plotted against sequence divergence using DAMBE (ver. 4.1.19) [28]. Prior to combining different gene fragments, we investigated the degree of heterogeneity between them using the partition-homogeneity test [29].

The data was subjected to three different phylogenetic analyses using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). ML and MP analyses were carried out with PAUP* v4.0b10 [30]. The MP method was performed using heuristic searches with 1000 random-addition sequence replicates, transitions and transversions were given equal weight. Consistency index (CI), retention index (RI), and rescaled consistency index were calculated for the resulting MP trees. ML analysis was performed using heuristic searches with 10 random-addition sequence replicates, based on the best substitution model, which was selected using the Akaike information criterion (AIC) [31], as implemented in the computer program Modeltest v3.7 [32]. Support for nodes of the resulting MP and ML trees was assessed by analyses of 1000 and 100 bootstrap replicates in PAUP* v4.0b10, respectively [33]. Clades with bootstrap values and posterior probabilities (see below) above 95 were considered to be well-supported. Finally, BI was carried out using MrBayes v3.1.2 based on the best substitution model [34]. To reduce the risk of the chain

being trapped on local optima [35,36], two runs were performed simultaneously with four Markov chains (three heated and one cold), starting from random trees. The Markov chains were run for 2000000 generations, and sampling every 100 generations thinned the data to 20000 sample points. The program Tracer v1.3 was used to determine when the log likelihood ($\ln L$) of sampled trees reached a stationary distribution [37]. Generations sampled before the chain reached stationary were discarded as burn-in, and the remaining trees were used to create 50% majority-rule consensus tree and to estimate Bayesian posterior probabilities (BPPs). The average p -distances of COI and 16S rRNA sequences between major clades were calculated using the program MEGA v3.1 [38].

2 Results

2.1 Sequence and tree statistics

The sequence data we obtained did not reach saturation. Of the 1148 bp in the combined alignment, 489 positions were variable and 386 were phylogenetically informative (42.6% and 33.6%, respectively), and among 386 informative positions, 196 and 190 characters were contributed by COI and 16S, respectively. Genetic distances (p -distance) for both genes are shown in Tables 2 and 3.

The GTR+G+I model was selected as the best-fitting model of nucleotide substitution. Settings for this model were as follows: R -matrix = (0.3434, 8.9517, 1.3819, 0.5061, 3.9593 and 1.0000); base frequencies = (A = 0.2961, C = 0.1199, G = 0.1796 and T = 0.4044); Proportion of invariable sites = 0.2924; and the shape-parameter of the γ distribution = 0.6243.

The twelve most parsimonious trees (MPTs) were obtained with 1132 evolutionary steps, a consistency index (CI) of 0.593 and a retention index (RI) of 0.849. The likelihood value of the ML tree was $\ln L = -7043.23$, and the likelihood values of the consensus tree in the Bayesian approach were -6925.65 and -6937.17 for the cold chain of runs 1 and 2, respectively. The first 1000 samples were discarded as burn-in and the remaining trees were used to construct the consensus tree.

Table 2 Genetic distances (uncorrected p -distances) for 16S rRNA^{a)}

	Myan	Mbic	Mman	Mekong	Cch	Clo	Mdian
Myan	0.004–0.012						
Mbic	0.129	–					
Mman	0.118	0.006	0–0.107				
Mekong	0.119	0.045	0.029	0.020			
Cch	0.108	0.099	0.087	0.108	0		
Clo	0.102	0.093	0.077	0.092	0.033	0	
Mdian	0.095	0.090	0.076	0.091	0.035	0.006	0–0.016

a) Myan, *Margarya yangtsunghaiensis*; Mbic, *Margarya bicotata*; Mman, *Margarya mansuyi*; Cch, *Cipangopaludina chinensis*; Clo, *Cipangopaludina longispria*; 'Mdian' was shared by species from Lake Dianchi and Haixihai reservoir; Mekong, *Mekongia* sp.

Table 3 Genetic distances (uncorrected *p*-distances) for COI

	Myan	Mbic	Mman	Mekong	Cch	Clo	Mdian
Myan	0.006–0.010						
Mbic	0.1565	–					
Mman	0.1541	0.0158	0–0.009				
Mekong	0.1629	0.0462	0.0449	–			
Cch	0.1574	0.1105	0.1125	0.1122	0.008		
Clo	0.1448	0.1100	0.1091	0.1139	0.0602	0.006	
Mdian	0.1408	0.1100	0.1100	0.1150	0.0645	0.0363	0–0.01

The partition-homogeneity test indicates no significant heterogeneity among the two gene fragments (*P*-value is 0.265). Therefore, the combination of the COI and 16S gene partitions should not be an issue.

2.2 Phylogenetic relationships

Three phylogenetic analyses of the combined dataset yielded identical topologies. Thus, only the BI tree is presented here (Figure 3). The following relationships were revealed by the three analyses:

The monophyly of *Margarya* is not supported, with species being found in three different clades (clades 1–3, Figure 3) that with one exception (Clade 1) also comprise representatives of other viviparid genera. The species of *Margarya* in these clades are also set apart by comparatively large genetic distances of at least 7.7% (16S) and 11.0% (COI; Tables 2 and 3).

Margarya yangtsunghaiensis (Clade 1) is basal and sister taxon to a clade comprising the remaining *Margarya* species plus *Cipangopaludina*, *Mekongia*, and *Sinotaia*.

The latter group consists of two highly supported clades. Clade 2 comprises *Margarya mansuyi* from Lakes Xingyun and Qilu, and *M. bicotata* from Lake Fuxian, which forms the sister group to a *Mekongia* and *Sinotaia* subclade. The *Margarya* subclade within Clade 2 is not highly supported except by BPP values.

Clade 3 comprises all *Margarya* species from Lake Dianchi and Haixihai reservoir, viz. *M. melanioides*, *M. tropidophora*, *M. monodi* and *M. bicotata*. This clade is not truly a *Margarya* clade, as it is not even monophyletic for the genus, the species of *Margarya* clustering indiscriminately with *Cipangopaludina dianchiensis* and *C. sp. Cipangopaludina chinensis* and *C. longispira* are also basal or sister, respectively, to all other taxa in Clade 3. The specimens of *M. melanioides* from Haixihai reservoir are nested within the Lake Dianchi samples, without forming a geographically defined sub-lineage.

At a lower taxonomic level, *Margarya bicotata* is not monophyletic: the specimen of *M. bicotata* from Lake Fuxian (type locality) is sister group to *M. mansuyi* in Clade 2 and distinct from those recognized previously as *M. bi-*

cotata from Lake Dianchi (Clade 3), which cluster indiscriminately with *M. melanioides*.

3 Discussion

The non-monophyly of *Margarya*, which is shown to comprise three molecular clades, is rather surprising given its rather conspicuous large and ornamented shell that has, until recently, clearly differentiated this genus from all other viviparid genera in East and Southeast Asia. Interestingly, a multiple origin of *Margarya* was already suggested by Anandale based on differences in transition series from smooth to sculptured shells in the fossil record [15]. The topology of the molecular tree suggests, however, that the comparatively simple shells of *Mekongia*, *Sinotaia* and *Cipangopaludina*, which are devoid of any sculpture, have been derived from the unique shell of *Margarya*, a surprisingly interesting, albeit difficult, fact to interpret. Particularly striking is the fact that species assigned to both *Margarya* and *Cipangopaludina* are included in Clade 3 without any support for the monophyly of either (Figure 3). This placement would imply multiple transitions between the two shell forms if the tree were interpreted literally. Shell differences are not only apparent in the adult shells of these two genera, where both share an almost equally large shell size. The embryonic shells also consistently differ, having nodules or spiral keels on the embryonic shell in *Margarya* as opposed to the three rows of chaetae in *Cipangopaludina* [39]. The shell may be subjected to environmental pressure and cases of phenotypic plasticity in shell morphology have been reported from various gastropod groups, albeit not from viviparids [20,40,41].

Anatomical data that might provide additional evidence with respect to the molecular results are not available at all for *Margarya*, and are at best fragmentary for the other Asian genera, *Cipangopaludina*, *Mekongia* and *Sinotaia*, for which our analyses suggest a close relationship to some of the *Margarya* clades [37,42]. Given this lack of data and the apparent confusion between genera stemming from the sole reliance on a shell-based delineation of taxa, a revision of *Margarya* and other Asian viviparid genera, particularly

Figure 3 Molecular phylogeny of *Margarya* based on Bayesian inference. The nodal numbers are BPP, ML and MP bootstrap values in percent. Only values above 50% are given. The numbered black vertical bars indicate the three major clades (see text) comprising species of *Margarya*, which are highlighted in bold type.

Cipangopaludina, *Mekongia* and *Sinotaia*, is clearly needed. This need is highlighted by our finding that the monophyly of *Cipangopaludina* is also not supported. *Cipangopaludina chinensis*, the type species of *Cipangopaludina*, and *C. longispira* are paraphyletic with respect to *Margarya* Clade 3, and both *C. dianchiensis* and *C. sp.* cluster with several *Margarya* species in that clade (compare above). The taxonomic confusion at the generic level we found are not exclusive to Asian viviparids, as a recent molecular study on African viviparids found that the characteristic genus *Neothauma* Smith, 1880 endemic to Lake Tanganyika, is not sister group to the only other African genus *Bellamya* Jousseaume, 1886, but rather to a subgroup of the latter [24].

Despite the obvious shortcomings of basing genera solely on shell characters, the shell nevertheless provides taxonomically important characters, even in the case of *Margarya* at an infrageneric level. Shu et al. suggested that *M. mansuyi* (including *M. bicotata* – Clade 2) and *M. melanioides* (Clade 3) occupy distinct morphospaces as inferred from size ratios [21]. The regular nodules on the entire shell and the pointed apex of *M. yangtsunghaiensis* (Clade 1) distinguish this taxon from all other species of *Margarya*, and the phylogenetic tree strongly supports this distinctiveness. Data from shell morphology can thus be regarded as compatible with the molecular results with respect to the differences between the three *Margarya* clades *per se*, but fail to account for the intergeneric confusion.

At present, drawing any taxonomic conclusion concerning generic assignments from the existing data seems premature. While the non-monophyly of *Margarya* hints at the possible existence of several genera, it is not possible to tentatively assign the name *Margarya* to Clade 3, which contains the type species, *M. melanioides*, as its sequences intermittently cluster with those of three other species of *Margarya* and two species of *Cipangopaludina*. Specimens from the type locality, which may shed light on this question, were not available at all, as *Margarya* is more likely than not extinct in Lake Er-hai.

The taxonomy of *Margarya* is obviously in need of revision at the species level. The only clear pattern at the intra-generic level is the geographic match of the three clades of *Margarya* with single lakes or lake complexes, viz. (1) Yangzonghai, (2) Fuxian-Xingyun-Qilu, and (3) Dianchi-Haixihai (compare Figure 1). However, with the exception of *M. yangtsunghaiensis* (Clade 1) and *M. mansuyi* (Clade 2), (sub) clades do not correspond to species of *Margarya* (Figure 3). This is most striking in Clade 3, where none of the six species occurring in Lake Dianchi and the Haixihai reservoir identified as *M. melanioides*, *M. tropidophora*, *M. monodi*, *M. bicotata*, and *Cipangopaludina dianchiensis* and *C. sp.* appears as monophyletic in the molecular tree. This topology and the low genetic distances (as evidenced by short branch length and low maximum *p*-distances of 1.6 and 3.2% for *16S* and *COI*, respectively)

within this clade indicate that the previously recognized six species from Lake Dianchi, *M. melanioides*, *M. tropidophora*, *M. monodi*, *M. bicotata*, *Cipangopaludina dianchiensis*, plus *C. sp.*, may be conspecific, even though it would remain to be seen in which genus. However, many factors can cause a gene tree to differ from a species tree [43], so we suggest investigating species limits in *Margarya* by using additional evidence, e.g. from highly variable nuclear markers such as *AFLPs* or microsatellites, before deciding on the synonymy of species within clades 2 and 3. It is important to keep in mind that widespread non-monophyly at the species level was also found by Sengupta et al., who also used nuclear markers, in African *Bellamya*, suggesting that this may be a common phenomenon in viviparids [24].

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