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Phylogenetic relationships and estimation of divergence times among Sisoridae catfishes

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Nineteen taxa representing 10 genera of Sisoridae were subjected to phylogenetic analyses of sequence data for the nuclear genes Plagl2 and ADNP and the mitochondrial gene cytochrome *b*. The three data sets were analyzed separately and combined into a single data set to reconstruct phylogenetic relationships among Chinese sisorids. Both Chinese Sisoridae as a whole and the glyptosternoid taxa formed monophyletic groups. The genus *Pseudecheneis* is likely to be the earliest diverging extant genus among the Chinese Sisoridae. The four *Pareuchiloglanis* species included in the study formed a monophyletic group. *Glaridoglanis* was indicated to be earliest diverging glyptosternoid, followed by *Glyptosternon maculatum* and *Exostoma labiatum*. Our data supported the conclusion that *Oreoglanis* and *Pseudexostoma* both formed a monophyletic group. On the basis of the fossil record and the results of a molecular dating analysis, we estimated that the Sisoridae diverged in the late Miocene about 12.2 Mya. The glyptosternoid clade was indicated to have diverged, also in the late Miocene, about 10.7 Mya, and the more specialized glyptosternoid genera, such as *Pareuchiloglanis*, originated in the Pleistocene (within 1.9 Mya). The speciation of glyptosternoid fishes is hypothesized to be closely related with the uplift of the Qinghai-Tibet Plateau.

Sisoridae, glyptosternoids, phylogenetic monophyly, speciation

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The cytochrome *b* (Cyt*b*) gene is a mitochondrial DNA (mtDNA) molecular marker whose structure and function is the best understood among 13 protein-coding mtDNA genes. Given its moderate rate of evolution and abundant sequence variability, Cyt*b* is used for effective intraspecific and interspecific identification, thus it is widely used in the study of phylogenetic relationships. With the depth of research and improvement in analytic methods, some potential problems exist with practical application of mitochondrial genes, such as diversity of molecular structure, paternal inheritance, nuclear copy insertion, mitochondrial genetic material exchange among allied species [1–3]. Not only could proteins encoded by nuclear genes be involved directly in expression and regulation of a mitochondrial gene, but also play an important role in maintaining the stability of mitochondrial genes. More reliable results can be obtained by using molecular markers together with combined mitochondrial and nuclear genetic data to identify species and answer questions related to species evolution and phylogenetic relationships [4].

The family Sisoridae, which belongs to the Siluriformes, was established by Regan in 1911 [5]. It is one of the largest and most diverse Asiatic families, and numerous sisorid species inhabit basins on the Qinghai-Tibetan Plateau and the East Himalayas [6]. Glyptosternoid fishes are a large natural group within the Sisoridae. Eight genera of glyptosternoid fishes are found in China, namely *Parachiloglanis*, *Glyptosternum*, *Euchiloglanis*, *Pareuchiloglanis*, *Glarido-*

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glanis, *Oreoglanis*, *Pseudexostoma* and *Exostoma*. The other genera of Sisoridae are *Pseudecheneis*, *Glyptothorax*, *Bagarius* and *Gagata*. The taxa are mainly distributed in the Yarlung Zangbo, Nujiang, Lancang, Jinsha, Irrawaddy, Pearl and Red Rivers, and the regional distribution areas include Tibet, Qinghai, Gansu, Sichuan, Sichuan, Guizhou, Guangxi, and especially Yunnan where the most abundant genera and species are distributed.

Hypothesized relationships within the Sisoridae, determined on the basis of morphology and molecular biology studies, remain controversial (Figure 1) [7–15]. A number of questions remain unresolved. For example, which is the most primitive glyptosternoid fish species, *Glyptosternum maculatum* or *Glaridoglanis andersonii*? Are *Oreoglanis* and *Pareuchiloglanis* monophyletic groups? What is the phylogenetic position of *Pseudecheneis* within the Sisoridae? In the present study we used sequence data for the Cyt*b* mitochondrial gene and two nuclear genes, ADNP and

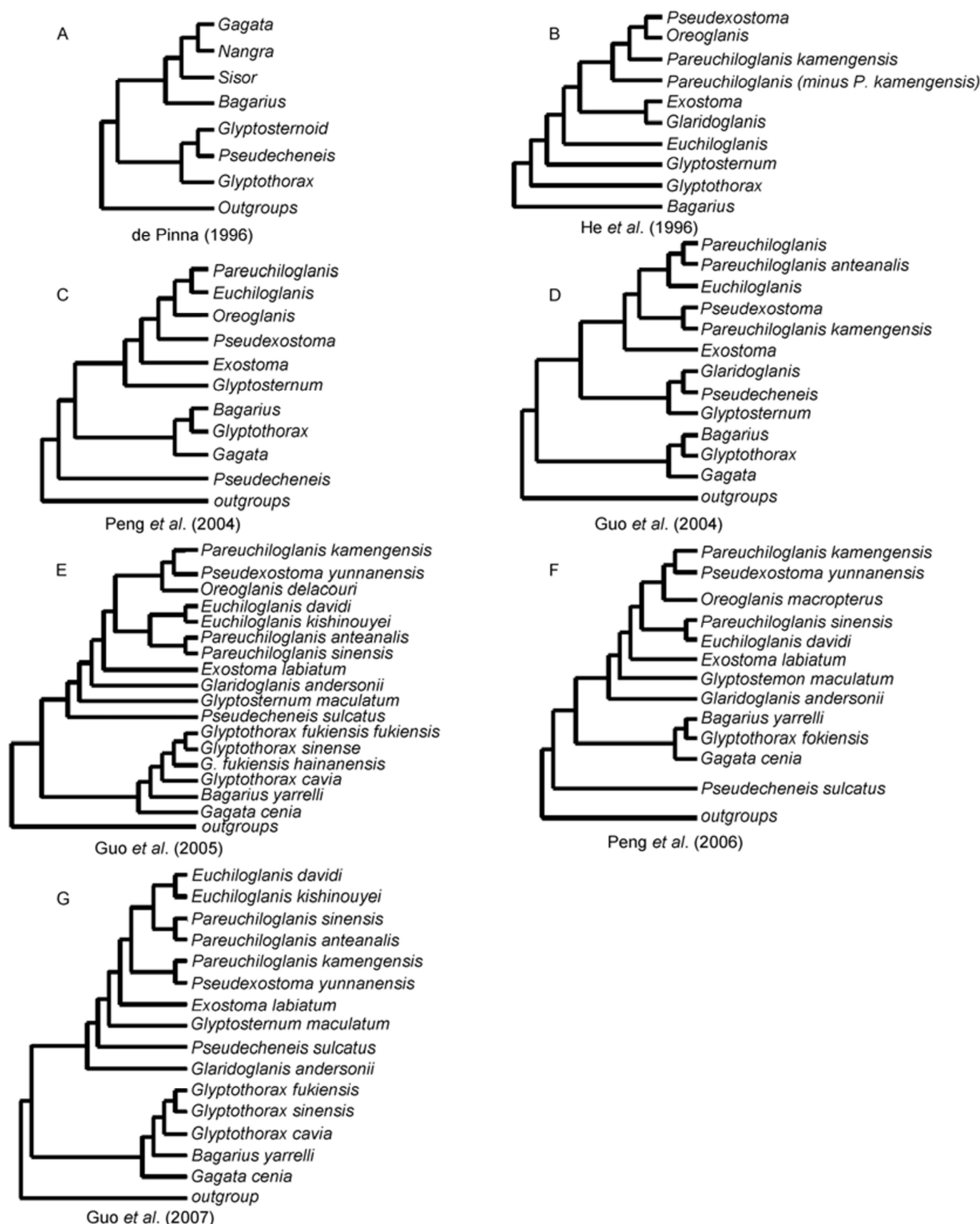

Figure 1 Previous phylogenetic reconstructions for glyptosternoid fishes.

Plagl2, to investigate phylogenetic relationships among Chinese Sisoridae. In addition, we used a molecular clock to estimate the species divergence times to explore further phylogenetic relationships among glyptosternoid fishes and the possible mechanisms of diversification.

1 Materials and methods

1.1 Materials

The Sisoridae specimens used in this study were collected from Yunnan, Tibet, Sichuan, Guangxi and Hunan, China. Muscle tissue from the specimens was preserved in 95% ethanol. The specimens were identified at the Institute of Hydrobiology, Chinese Academy of Sciences, in accordance with the Siluriformes treatment in the *Fauna Sinica Osteichthyes* [16]. Specimens were stored in the Department of Fish Phylogenetics and Biogeography, Institute of Hydrobiology, Chinese Academy of Sciences.

The specimens used in this study consisted of 11 genera of Sisoridae (Table 1), which included seven genera of glyptosternoid fishes, namely *Glaridoglanis*, *Glyptosternum*, *Euchiloglanis*, *Pareuchiloglanis*, *Exostoma*, *Oreoglanis*, and *Pseudexostoma*. *Parachiloglanis* was not included owing to a lack of samples. Representatives of four genera of non-glyptosternoid fishes (*Bagarius*, *Gagata*, *Pseudecheneis*, and *Glyptothorax*) were sampled. *Liobagrus nigricauda* (from the family Amblycipitidae) was included as the outgroup.

1.2 PCR amplification, cloning and sequencing

Total genomic DNA was extracted from ethanol-preserved fish muscle using a phenol/chloroform extraction method, dissolved with sterile water, then stored at –4°C. The primers used to amplify the Plagl2 and Cyt*b* genes are listed in Table 2. ADNP (activity-dependent neuroprotector homeobox) is a single-copy gene in the zebrafish genome. We identified orthologous genes in the genome of pufferfish (fugu) and medaka, and designed primers to amplify the gene in Sisoridae.

Table 1 Sisoridae samples used for gene sequencing and phylogenetic analysis in this study[a)]

Family	Genus	Taxon	GenBank accession number		
			Plagl2	ADNP	Cyt*b*
Amblycipitidae	*Liobagrus*	*Liobagrus nigricauda*	JN986989	JN986965	JN986968
Sisoridae	*Pseudecheneis*	*Pseudecheneis sulcatus*	JN986988	JN986964	DQ192469
Sisoridae	*Bagarius*	*Bagarius yarrelli*	JN986972	–	AF416897
Sisoridae	*Gagata*	*Gagata cenia*	–	–	DQ192468
Sisoridae	*Glyptothorax*	*Glyptothorax fukiensis honghensis*	JN986984	JN986957	–
Sisoridae	*Glyptothorax*	*Glyptothorax trilineatus*	JN986983	–	JN986967
Sisoridae	*Glyptothorax*	*Glyptothorax fukiensis fukiensis*	JN986985	JN986956	–
Sisoridae	*Glyptothorax*	*Glyptothorax cavia*	JN986986	–	JN986969
Sisoridae	*Glyptosternum*	*Glyptosternum maculatum*	JN986978	JN986959	DQ192471
Sisoridae	*Exostoma*	*Exostoma labiatum*	JN986981	–	DQ192461
Sisoridae	*Glaridoglanis*	*Glaridoglanis andersonii*	JN986974	–	AY601769
Sisoridae	*Pareuchiloglanis*	*Pareuchiloglanis gracilicaudata*	JN986976	JN986954	–
Sisoridae	*Pareuchiloglanis*	*Pareuchiloglanis feae*	–	JN986963	JN986971
Sisoridae	*Pareuchiloglanis*	*Pareuchiloglanis kamengensis*	JN986973	JN986960	DQ192477
Sisoridae	*Pareuchiloglanis*	*Pareuchiloglanis gongshanensis*	JN986975	JN986953	JN986970
Sisoridae	*Euchiloglanis*	*Euchiloglanis davidi*	JN986977	JN986961	DQ192485
Sisoridae	*Oreoglanis*	*Oreoglanis macropterus*	JN986979	JN986962	DQ192479
Sisoridae	*Oreoglanis*	*Oreoglanis delacourii*	JN986980	JN986955	JN986966
Sisoridae	*Pseudexostoma*	*Pseudexostoma yunnanensis yunnanensis*	JN986982	JN986958	DQ192478

a) "–" indicates that no sequence was obtained.

Table 2 Primer sequences used for gene amplification

Target gene	Primer	5′–3′ sequence	Reference
Plagl2	Plagl2_F9	CCACACACTCYCCACAGAA	[17]
	Plagl2_R930	TTCTCAAGCAGGTATGAGGTAGA	
	Plagl2_F51	AAAAGATGTTTCACCGMAAAGA	
	Plagl2_R920	GGTATGAGGTAGATCCSAGCTG	
Cyt*b*	L14724(cytbF)	GACTTGAAAAACCACCGTTG	[18]
	H15915(cytbR)	CTCCGATCTCCGGATTACAAGAC	

The Cyt*b* and ADNP genes were amplified in a 50 μL reaction volume, which contained 5 μL 10×PCR buffer (TaKaRa), 4 μL 2.5 mmol L^{-1} dNTPs (TaKaRa), 2 μL 10 mmol L^{-1} of each primer, 2.5 U Taq DNA polymerase (5 U μL^{-1}; TaKaRa), 1.0 μL template DNA (approximately 40 ng), and sterile water to bring the total volume to 50 μL. The PCR protocol was as follows: denaturation at 94°C for 4 min, then 31 cycles of denaturation at 94°C for 30 s, annealing at 54–56°C for 30 s, and elongation at 72°C (for 1.5 min for Cyt*b*, 1 min for ADNP), a final extension at 72°C for 10 min, and storage at 4°C.

We used nested PCR to amplify the Plagl2 gene. The first round of amplification was performed in a 10 μL reaction volume that contained 1 μL 10× PCR buffer (TaKaRa), 0.5 μL of 2.5 mmol L^{-1} dNTPs (TaKaRa), 0.5 μL of each primer (10 mmol L^{-1}), 0.1 μL rTaq enzyme (5 U vL^{-1}; TaKaRa), 0.5 μL template DNA (about 40 ng), and sterile water to bring the total volume to 10 μL. The second round of amplification was performed in a 50 μL reaction solution, which had the same composition as that used for Cytb and ADNP amplification except that the template was the first-round PCR products. The reaction conditions followed Li *et al*. [17].

The PCR products were purified with a gel extraction kit (OMEGA). A portion of the purified products were used for sequencing, and a portion was used for cloning; the purified products for which sequencing was unsuccessful needed to be cloned and then sequenced. The PMD18-T vector (TaKaRa) and *Escherichia coli* Top10 (Invitrogen) were used in cloning. The samples were sequenced by Beijing Tianyi Huiyuan.

1.3 Data analysis

1.3.1 Sequence matching and alignment

The sequences were analyzed with the Lasergene version 7.0 (DNASTAR) software package, of which Seqman was used for sequence assembly into contigs, and MegAlign was used to construct a multiple alignment of the sequences. The sequence alignment was adjusted manually with Clustal W software [19]. The nucleotide gene sequences were translated into amino acids with MEGA 4.0 software [20] to ensure that no stop codons were inserted in the target fragment during the sequencing procedure. We did removement and addition to avoid a single base insertions or deletion due to sequencing through multiple sequencing and alignment, and a BLAST search in the GenBank database (NCBI) was performed to ensure that the whole sequences amplified from the nuclear genes were the target products.

1.3.2 Phylogenetic analysis

Four data sets were analyzed: three single-gene (Cytb, ADNP and Plagl2) data sets and a 'total evidence' data set in which all data was combined. Maximum likelihood and Bayesian inference to reconstruct phylogenetic trees from the sequence alignments. Modeltest 3.7 software [21] was used to determine the most appropriate model of sequence evolution. A GTR+I+G model was selected as the optimal substitution model for the phylogenetic analyses. We use the combined sequences sets and use the following partition strategies: protein-coding gene Cytb and nuclear genes (ADNP and Plagl2) with nine partitions of the 1st, 2nd, 3rd codon position. Bayesian inference was performed with MrBayes 3.0 software [22] with and without partitions. Analyses were run for 2×10^7 generations and sampled every 1000 generations to ensure its independence. Four chains were run simultaneously, comprising three hot chains (T=0.05) and one cold chain, and 2500 trees were discarded as 'burn in'. RAxML [23] analysis was run for 1000 bootstrap replicates with an unpartitioned GTR+G model.

1.3.3 Divergence time estimation

A rate consistency test was used for analyzing the evolutionary rate of each branch in the phylogeny (Figure 3). The substitution rate of the various branches was not consistent, therefore a strict molecular clock was not supported ($P<$ 0.01). Consequently, MULTIDISTRIBUTE software was used to estimate the divergence time of each branch. This software performs a combined analysis with PAML [24] and MULTIDIVTIME [25]. MUTIDIVTIME permits a restriction to be placed on the minimum and maximum dates of the fossil record of each calibration point. The first date of appearance in the fossil record is the minimum date, whereas the maximum date is more difficult to estimate. Using the fossil record of the genus *Bagarius* (5.6 Mya) [26] as a calibration point, the differentiation time of glyptosternoid fishes was estimated with the combined mitochondrial gene and two nuclear genes data.

The Markov chain was run for 10^7 generations with sampling every 100 generations, and the first 10000 cycles were discarded as 'burn in'. Before running the analysis, the age of the root node of the phylogenetic tree, the substitution rate and branch independent rate χ±SE were determined. A date of 12 Mya was set as the expected divergence time of the basal node to end point node of the phylogenetic tree, and the divergence time was not constrained on each node of phylogenetix tree except the position of genus *Bagarius*. Every 100 years was considered as a time unit (rttm=1.2, rttmsd=0.12). The average a priori rate for the root node was 0.173, and the standard deviation of the a priori rate for the root was 1.173. The mean and standard deviation of the a priori Brownian motion constant were both 0.83.

2 Results and discussion

2.1 Phylogenetic relationships of Chinese Sisoridae catfishes

Single-gene data sets (ADNP, Cyt*b*, and Plagl2) were used

to construct phylogenetic trees with PhyML. Sisoridae fishes were monophyletic, among which glyptosternoid fishes formed a monophyletic group (Figure 2). *Pseudecheneis sulcatus* was placed as an early divergence among Sisoridae fishes. *Glyptothorax*, *Oreoglanis* and *Pareuchiloglanis* were all indicated to be monophyletic. Each glyptosternoid fish genus was located in a different position in the phylogenies derived from the different genes. In the ADNP phylogenetic tree (Figure2 A), under the conditions of lacking samples of *Glaridoglanis andersonii*, *Euchiloglanis davidi* and *Glyptosternum maculatum* formed a sister group, whereas in the Cyt*b* phylogeny, *Glyptosternum maculatum* was indicated to be the earliest divergence among glyptosternoid fishes, followed by *Glaridoglanis andersonii*, *Exostoma labiatum* (Figure 2B). The Plagl2 data set indicated *Glaridoglanis andersonii* was the earliest divergence among the glyptosternoid fishes, followed by *Glyptosternum maculatum* and *Euchiloglanis davidi* (Figure 2C). *Exostoma labiatum* and *Pseudexostoma yunnanensis yunnanensis* were sister taxa in the Plagl2 phylogeny. Thus, the topology of the phylogenetic trees differed when constructed from the different sequence data sets. Therefore, it is important to determine which gene provides the most reliable information for reconstruction of phylogenetic relationships among glyptosternoid fishes.

Analysis of the combined data set (Cyt*b*, ADNP and Plagl2) with different phylogenetic reconstruction methods yielded phylogenetic trees with a consistent topology and high posterior probabilities (Figure 3). *Pseudecheneis sulcatus* was the earliest-diverging species in the Sisoridae, which was consistent with some previous research [14,15]. Glyptosternoid fishes and the other three Sisoridae genera (*Gagata, Bagarius* and *Glyptothorax*) both formed monophyletic groups with a sister relationship. The four representative species of *Pareuchiloglanis* also formed a monophyletic group, whereas most previous studies did not support a monophyletic *Pareuchiloglanis* [12,13,15,27]. The probable reason for the discrepancy is differences in the representative species selected. In previous studies *Pareuchilognis* was represented mainly by *P. kamengensis*, *P. sinensis* and *P. anteanalis*, whereas in the current analysis *P. kamengensis*, *P. feae*, *P. gongshanensis* and *P. gracilicaudata* were used. The relationships of the species on phylogenetic tree were probably inflected by different species selected and different markers used. The phylogenetic reconstructions obtained in the present study indicated *Glaridoglanis andersonii* was an early divergence among glyptosternoid fishes, which was consistent with phylogenetic reconstructions by Peng *et al*. [14,15] derived from Cyt*b* and ND4 sequence data, and analysis of *rps*7 se-

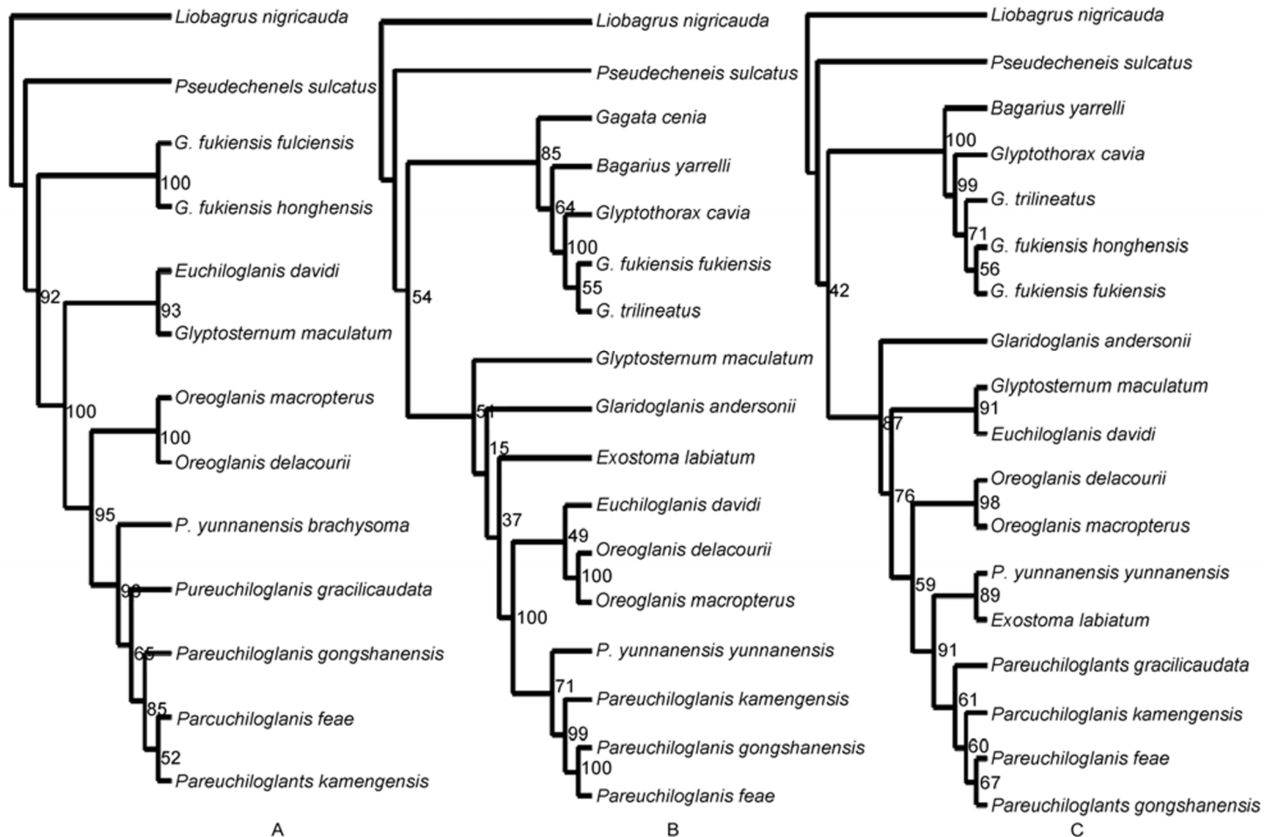

Figure 2 Phylogenetic relationships of Chinese Sisoridae inferred from analysis of single gene data sets. A, ADNP. B, Cyt*b*. C, Plagl2. Branch support values at each node were obtained from a PhyML analysis with 1000 bootstrap replicates.

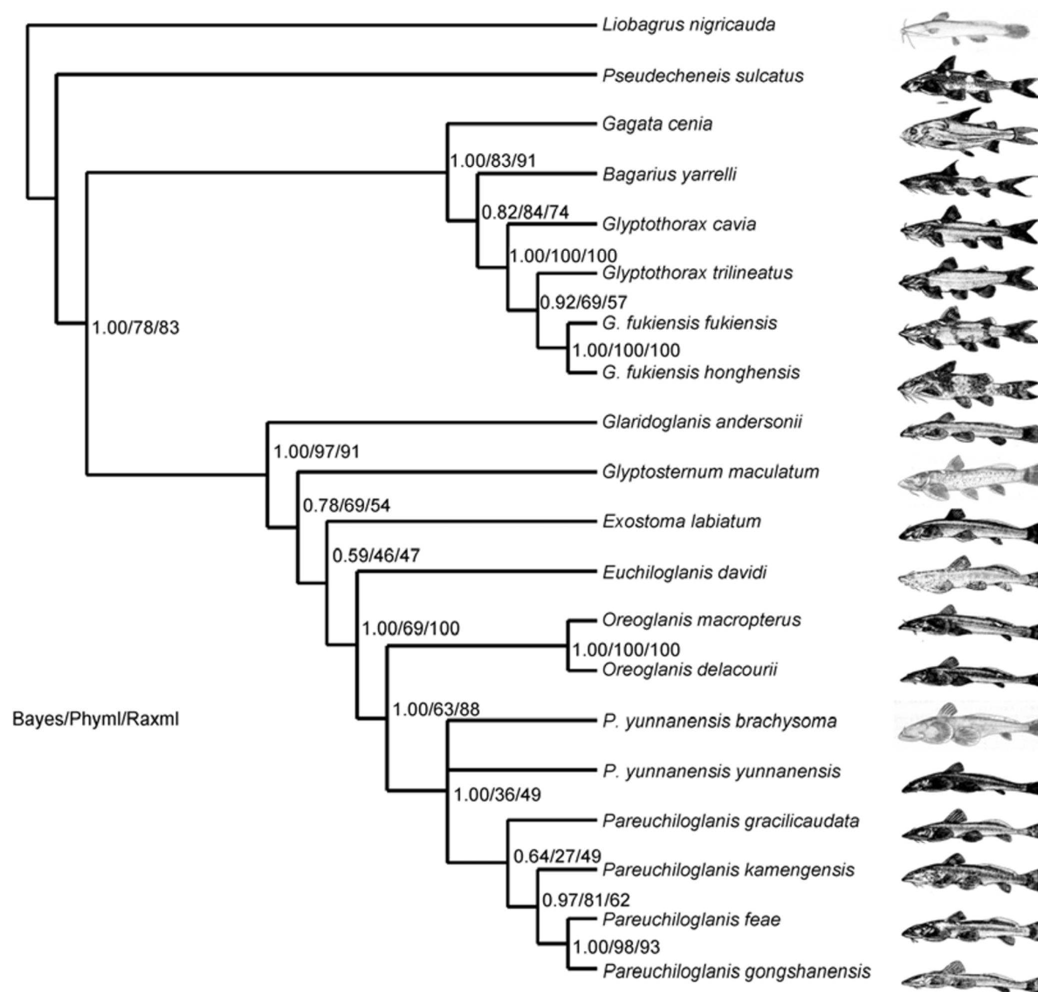

Figure 3 Phylogenetic relationships of Chinese Sisoridae inferred from analysis of the combined data set comprising the Cyt*b*, ADNP and Plagl2 sequence data constructed by Bayesian inference. Branch support values at each node were obtained from unpartitioned Bayesian inference, PhyML, and RaxML analyses, respectively.

quences by Guo *et al*. [13]. However, this finding differed from the relationship suggested in a phylogenetic analysis of a combined Cyt*b* and 16S rRNA data set by Guo *et al*. [12], and also showed some differences from He's [11] morphological phylogenetic analysis of 60 skeletal characters.

The genus *Pseudecheneis* has been placed in conflicting positions in previous phylogenetic reconstructions of Sisoridae. Peng *et al*. [14,15] chose *Pseudecheneis sulcatus* as a representative species of the genus, and found that it was indicated to be an early divergence in the diversification of Sisoridae fishes. De Pinna [7] and Kong [28] considered that *Pseudecheneis* and glyptosternoid fishes formed a sister group, whereas Guo *et al*. [12,13] found that *Pseudecheneis* was placed among the glyptosternoid fishes in phylogenetic trees. In the present study, *Pseudecheneis sulcatus* was consistently placed as the earliest divergence among Sisoridae fishes, which was consistent with previous studies by Peng *et al*. [14,15]. The difference in findings might reflect differences in the marker genes and analytic methods selected, in which case it is particularly important to choose a method to study phylogenetic and evolutionary relationships among Sisoridae fishes that utilizes morphological and molecular data combined.

A consensus on which is the most primitive extant species of glyptosternoid fish has not been reached. Previous morphological studies, such as those by He [11] and Kong [28], and molecular phylogenetic studies, such as Guo *et al*. [12], consistently indicate that *Glyptosternum maculatum* is the most primitive glyptosternoid species, whereas other recent studies [13,15] suggest the most primitive species is *Glaridoglanis andersonii*. Analysis of the combined Cyt*b*, ADNP and Plagl2 data set in the present study supported *Glaridoglanis andersonii* as being the most primitive species. In addition to the different methods of analyzing morphological and molecular data, marker genes also have an important impact on the outcome. Although an increasing number of marker genes are being developed to explore

the phylogenetic relationships between species and genera, limitations exist and not all phylogenetic relationships of a specific taxon can be resolved necessarily by one or a few marker genes. Combination of multiple research strategies will help to resolve phylogenetic and evolutionary relationships for a specific taxon.

In his study on whether the genus *Pareuchiloglanis* has been a monophyletic group since glyptosternoid fishes, on the basis of 60 skeletal characters, He [11] found that *Pareuchiloglanis* was non-monophyletic only when *Pseudecheneis* and *Oreolanis* were included in the data set. *Pareuchiloglanis* was not included in the study by de Pinna [7]. Guo *et al*. [13] found that the species of *Pareuchiloglanis*, *Euchiloglanis* and *Pseudexostoma* collectively formed a monophyletic group, whereas Guo *et al*. [12] showed that *Pseudexostoma*, *Oreolanis*, and *Pareuchiloglanis* formed a monophyletic group. Peng *et al*. [15] found that *Pareuchiloglanis* together with *Pseudexostoma*, *Oreolanis* and *Euchiloglanis* constitute a monophyletic group. The representatives species of *Pareuchiloglanis* included in the present study formed a monophyletic group in analyses of the combined data.

The morphological diversity of Sisoridae fishes and their specialized ecological requirements made them become made have a popular subject of morphological and molecular research. The present results, in comparison with those of previous studies, have permitted the following conclusions to be drawn.

(i) Sampling could affect a phylogenetic reconstruction. Complete sampling could make the phylogenetic tree close to real situation. While incomplete sampling always make phylogenetic tree different even the same analysis methods is used. In practical study, some species are difficult to obtain. Therefore, differences in the representative species selected can result in different topologies of phylogenetic tree. Inclusion and exclusion of certain representative species can result in phylogenetic reconstructions with different topologies [12,29].

(ii) Many genes are useful in diverse taxonomic groups, but the taxonomic level of utility depends on the level of variability (and thus informativeness)—the less variable, the higher the taxonomic level at which a region will be informative.

(iii) Different analytic methods can affect the topological structure of phylogenetic trees, especially interspecific relationships and the position of species in the phylogeny. The phylogenetic placement of a specific taxon using the same sequence data may not be consistent between different analytic methods, owing to the different algorithms, assumptions and methodologies employed.

Thus single factors may markedly affect the results of phylogenetic studies, but when these factors are combined the influence may be even greater. If different representative species, gene regions and different analytic methods are selected, incongruence between the phylogenetic trees may be stronger. By repeated trials and improvements, and careful selection of the representative species, it may be possible to establish the specific gene regions and analytic methods that are best suited to address specific research questions.

2.2 Molecular clock estimates and evolutionary analysis

The divergence time of each species of Sisoridae fishes is definitely shown in Figure 4. Sisoridae fishes were indicated to have originated in the late Miocene (about 12.2 Mya). Glyptosternoid fishes were indicated to have diverged in the late Miocene (about 10.7 Mya). The genera *Gagata* diverged about 8.9 Mya, and *Bagarius* diverged approximately 8.1 Mya. The divergence time of genera *Glyptothorax* was 4.5 Mya. The most primitive glyptosternoid fish species mainly originated in the late Miocene: the genera *Glaridoglanis* originated about 9.4 Mya, *Glyptosternum* diverged about 8.8 Mya, and *Exostoma* diverged about 8.3 Mya. The divergence time of more derived genera more occurred recently and was mainly concentrated after the Holocene (less than 4.4 Mya). For example, the generea *Euchiloglanis* diverged approximately 4.4 Mya, while genera *Pseudexostoma* and *Pareuchiloglanis* diverged about 2.3 Mya.

During the period 7–12 Mya, a number of the extant genera within the Sisoridae were indicated to have rapidly diverged, namely *Pseudecheneis*, *Gagata*, *Bagarius*, *Glyptosternum*, *Glaridoglanis*, and *Exostoma*. During the period 0.1–7 Mya, differentiation of species within genera was more prevalent; the genera *Pareuchiloglanis* and *Glyptothorax* diversified and gave rise to high species richness, especially in *Glyptothorax*, which exhibits the greatest species diversification and widest distribution of any genus of Sisoridae.

2.3 Relationships between origin and diversification of glyptosternoid fishes and uplift of the Tibetan Plateau

Glyptosternoid fishes are distributed over an extensive geographic area. From Guizhou and Guangxi in the east, northwards to Tibet and Sichuan, southwards to Laos and Vietnam, and westwards to India and Sumatra. The earliest fossil record of glyptosternoid fishes is in the Pliocene to Miocene from the Outer Himalayas—the Siva Cleveland Mountains (Siwalik Hills) [30]. Our molecular dating analysis indicated that *Bagarius yarrelli* diverged in the late Miocene, and divergence of glyptosternoid fishes also occurred during the late Miocene about 10.7 Mya. Our estimation was similar to that of Hora [6], who estimated that glyptosternoid fishes originated 9.8 Mya (95% confidence interval 8.2–11.0 Mya), but slightly earlier than that of Peng *et al*. [15] (about 6–8 Mya), and later than the estimation by Guo *et al.* [12] (19–24 Mya). On the basis of the existing fossil record of *Bagarius*, Chu [31] inferred that glyptosternoid fishes possibly diverged in the late Pliocene.

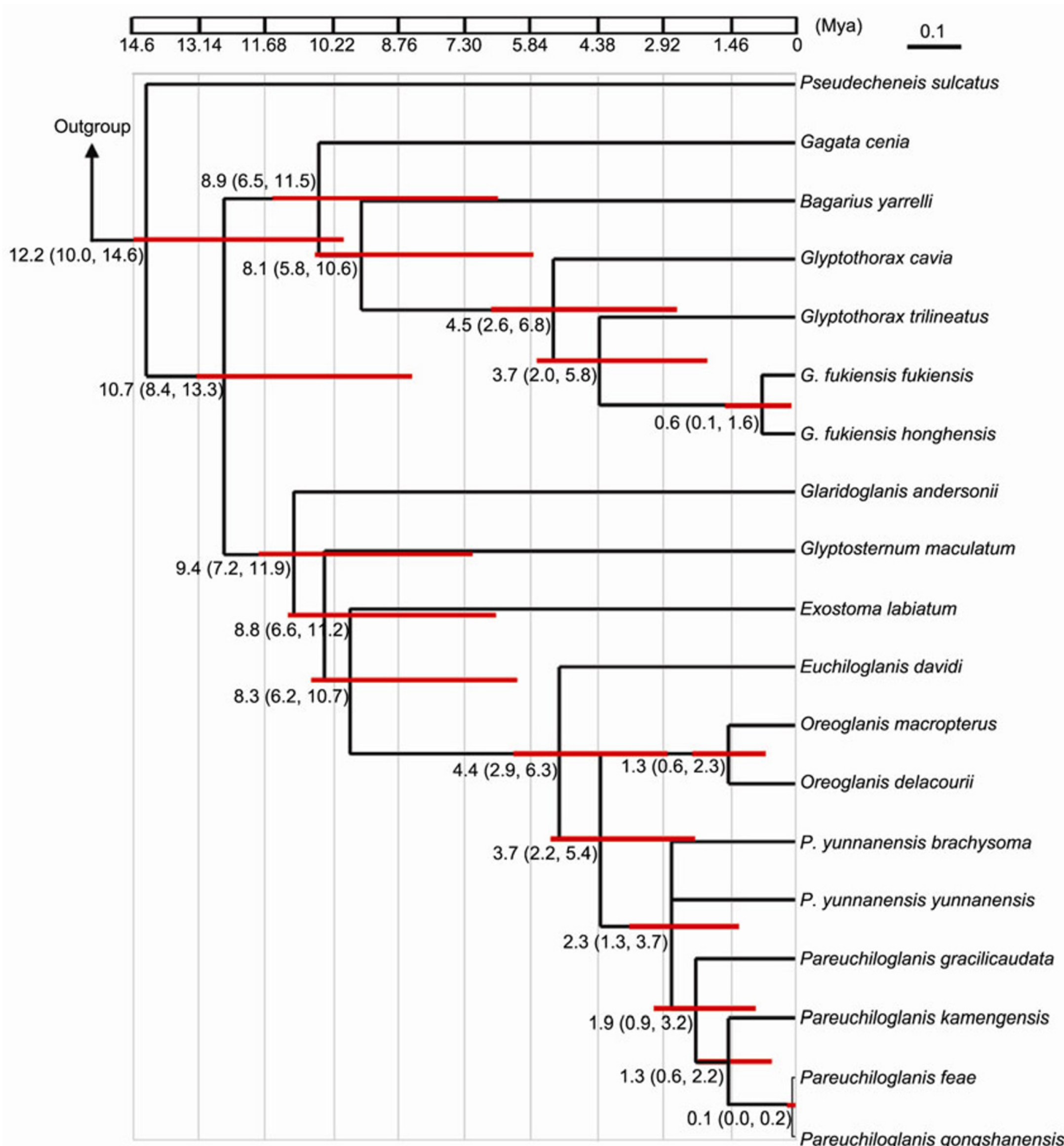

Figure 4 Estimated divergence times of Chinese Sisoridae. The tree topology was derived from a MULTIDIVTIME analysis of 19 Chinese Sisoridae and is consistent with the Bayesian inference tree presented in Figure 3. Branch lengths are proportional to the divergence times. The values at each node are the mean estimated divergence time (in Mya) and the 95% confidence interval (values in parentheses).

Specialized diversification of glyptosternoid fishes, such as within the genera *Glyptothorax*, *Pareuchiloglanis*, *Oreoglanis* and *Pseudexostoma*, occurred relatively recently with most species estimated to have diverged within 4.5 Mya. Between the Pliocene and Pleistocene (beginning at 5 Mya and peaking after 3.0 Mya), the Qinghai-Tibet Plateau was in a stage of rapid uplift. At present, the Qinghai-Tibet Plateau underwent substantial uplift, and until the early Pleistocene uplift of the plateau reached 2000–3000 m. The uplift of the plateau created riffle environments in the mountain river system, and under the stress of this environmental factor species diversification within *Glyptothorax* occurred, and Sisoridae are still undergoing rapid diversification [32]. Given that interactions between species and their environment are constant, we infer that the diversification of glyptosternoid fishes was closely related with the uplift of the Qinghai-Tibet Plateau. This inference is also supported by previous research on glyptosternoid fishes [31,33,34].

3 Conclusion

In this study we analyzed a combined sequence data set that comprised the mitochondrial gene Cyt*b* and nuclear genes ADNP and Plagl2. The Cyt*b* gene has evolved more rapidly than the nuclear genes, and thus the genes are most informative at different taxonomic levels. We obtained a consistent topological structure of phylogenetic tree with high

statistical support with different analytic methods. Owing to the limited availability of species samples, it was not possible to sample all species of glyptosternoid fishes. With increased sampling of representative species and combined analysis of morphological, molecular and ecological data, phylogenetic reconstructions for Sisoridae should become more complete and reflect the true taxonomic relationships and evolutionary history of Sisoridae fishes, which will provide the foundation to understand the origins and diversification of Sisoridae in the highlands of China.

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