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Cyprininae phylogeny revealed independent origins of the Tibetan Plateau endemic polyploid cyprinids and their diversifications related to the Neogene uplift of the plateau

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Origin and diversification of the Tibetan polyploid cyprinids (schizothoracins) may help us to explore relationships between diversification of the cyprinids and the Tibetan Plateau uplift. Cyprininae phylogeny was analyzed using mitochondrial and nuclear DNA sequences to trace origins of polyploidy and diversifications of schizothoracins. Ancestral states reconstruction for ploidy levels indicated that the Cyprininae was diploid origin and the schizothoracin clades tetraploid origins. There were two diversification rate shifts along with diversification of the cyprinine fishes in response to the Tibetan uplift. The unusual diversification shifts were located to branches subtending the clades of Tibetan polyploid cyprinids. Our analyses suggested that (i) phylogeny of Cyprininae recovered two independent origins of the Tibetan polyploidy schizothoracins; (ii) diversifications of the schizothoracins were closely related to the Neogene uplift of the Tibetan plateau in the following ways: the relatively ancient Late Oligocene-Middle Miocene adaptive radiation may be associated with the uplift of the southern Tibet and Himalaya; the Middle Miocene-Early Pleistocene lineage-specific diversification broadly coincident with major phase of the Neogene Tibetan uplift; and the most recent Pleistocene diversification shift in *Schizothorax* closely coincident with the successive Kunlun-Huanghe and Gonghe movements of the Tibetan uplift and the glaciation-induced climate oscillations on the plateau.

Cyprininae, polyploid, schizothoracins, the Tibetan Plateau, diversifications

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INTRODUCTION

The Tibetan Plateau, also known as the Qinghai-Tibetan Plateau, is the biggest and highest plateau with an area of 2.5 million square kilometers and an average elevation of over 4,500 m. The extreme environmental conditions characteristic of the Tibetan Plateau include high absolute elevation, low temperature, and hypoxia. Polyploidy in plants of the Tibetan Plateau is expected to occur at a high rate because of the tremendous species diversity of polyploidy endemic to the Plateau and the extensive habitats for alpine plants in the Plateau (Liu, 2004; Nie et al., 2005; Tang et al., 2005; Zhang et al., 2009), indicating that polyploidy is a common and successful evolutionary transition under the most extreme environmental conditions of the Tibetan Plateau. The extensive occurrence of polyploidy was also found in some groups of cyprinid fishes (Leggatt and Iwama, 2003; Yu et al., 1987, 1989), most of which were characteristic of the Tibetan Plateau and its adjacent regions (Yue, 2000). Although it is well known that cyprinid fishes endemic to the Tibetan Plateau and its adjacent regions are major vertebrates in which polyploidy can be commonly

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observed and expected, less attention has focused on origin and evolution of these polyploid cyprinids related to the Tibetan Plateau.

The carp family (Cyprinidae) is the largest freshwater fish family with an estimated 2,420 species (Nelson, 2006). The subfamily Cyprininae *sensu* Howes (Howes, 1991) (tribe Barbine (Chen et al., 1984; Chen, 1998)) approximately comprises 87 genera and 1,332 nominated species, which accounts for more than 50% of total cyprinid fishes and is distributed from southern Eurasia to Africa (Rainboth, 1991; Skelton et al., 1991). Morphologically, Cyprininae includes four subgroups, such as barbine, cyprinine, labeonine and schizothoracine, referred as four subfamilies by Chen (Chen, 1998). Wild species of Cyprininae fish vary greatly in ploid levels ranging from diploids (2*n*=50) to high polyploids (2*n*=417–470) (Arai, 2011; Buth et al., 1991; Leggatt and Iwama, 2003; Yu et al., 1987, 1989). Examples of polyploidy are commonly observed in barbine, especially in cyprinine and schizothoracine. To our knowledge, all examined schizothoracine species are polyploidy, but no occurrence of polyploids has been observed in labeonine fishes. Furthermore, all the currently recognized schizothoracine species is clustered on the Tibetan Plateau and its adjacent regions.

The major geological and palaeoclimatical events including uplift of the Tibtan Plateau and evolution of Monsoons have a great influence on environment in East Asia. It is supposed that uplift of the Tibetan Plateau was particularly relevant to the endemic organisms because the environmental and habitat variations were severely affected by the uplift of the plateau and the associated climatic changes. The Tibetan Plateau and the Himalayas were created by ongoing tectonic collision of the Indian Plate with the Eurasian Plate that started around 50 million years ago. Although details of the mechanism, amplitude, and timing responsible for the present Tibetan topography still remain unclear (Li and Fang, 1999; Tapponnier et al., 2001), further significant increases in altitude of the Tibetan plateau are thought to have occurred about 10–8 million years (Myr) ago (Harrison et al., 1992; Molnar et al., 1993), or more recently (Li and Fang, 1999; Zheng et al., 2000). The age estimation of schizothoracine and glyptosternoid suggested that diversification of fishes in the Tibetan Plateau and its adjacent regions occurred during the period of late Cenozoic uplift of the Tibetan Plateau. Molecular dating analyses of other metazoan groups on the Tibetan Plateau also indicated that environmental and habitat heterogeneity associated with uplift of the Tibetan Plateau in the Cenozoic had promoted speciation (Guo and Wang, 2007; Luo et al., 2004). Therefore, impacts of the late Cenozoic uplift of the Tibetan Plateau on speciation and distribution of organisms on this plateau have been well documented in studies concerning phylogeny and biogeography, offering alternative tests for the hypotheses of historical processes of geological and concomitant ecological variations on the Tibetan Plateau during the Cenozoic era. With the fact of distribution of many endemic fishes restricted or related to the Tibetan Plateau (including its adjacent regions) and the complexity of ploid levels, the subfamily Cyprininae presents a significant opportunity to explore the pattern of timing and tempo in taxonomic diversification of polyploid cyprinids correlated with the uplift of the Tibetan Plateau. Few studies, however, have examined the diversification patterns of the Cyprininae, in which polyploidy extensively occurred, in the context of uplift of the Tibetan Plateau in the Cenozoic era.

Using the molecular evidences in the present study, we investigated the impact of the uplift of the Tibetan Plateau and the associated climate change on the diversification of the subfamily Cyprininae, a species-rich group of polyploidy cyprinids. Because of the trouble of using molecular data in phylogenetic analyses of polyploid species (Rousseau-Gueutin et al., 2009), a method recommended to reduce the negative effect of polyploidy on reconstructing evolutionary history is to use the low-copy nuclear genes. Furthermore, phylogenetic analyses of mitochondrial and/or low-copy nuclear genes have shed light on the evolution of polyploids (Brysting et al., 2007; Evans et al., 2004, 2005; Fortune et al., 2007; Machordom and Doadrio, 2001; Marhold and Lihová, 2006; Sang, 2002; Tsigenopoulos et al., 2002). The nuclear recombination activating gene 2 (*RAG2*) encodes components of the recombinase involved in recombination of immunoglobin and T-cell receptor genes and appears as conserved single copy in zebrafish (Willett et al., 1997). Recently, the nuclear *RAG2* gene has been demonstrated to be phylogenetically informative in many phylogenetic analyses in Cyprinidae (Chen et al., 2015; Li et al., 2016; Wang et al., 2013; Yazdani Moghaddam et al., 2013). Therefore, we use the nuclear *RAG2* gene and the mitochondrial 16S ribosomal RNA (*16S rRNA*), cytochrome c oxidase subunit I (*COI*), NADH dehydrogenase subunit 4 (*ND4*) and cytochrome b (*cytb*) genes to reconstruct phylogeny of extant fishes of the subfamily Cyprininae. The aims of this study are (i) to provide the well resolved phylogeny of Cyprininae to understand the relationships among the polyploidy schizothoracins; (ii) to provide insights into the dates of cladogenetic events leading to origins and diversifications of the polyploid species within the subfamily Cyprininae; (iii) to determine whether the late Cenozoic uplift of the Tibetan Plateau has significantly affected the diversification of the plateau polyploid cyprinids by estimating patterns of diversification within the subfamily Cyprininae (Figure 1).

RESULTS

Phylogenetic analyses

The maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses of the combined DNA sequence data resulted in highly resolved, well supported, and compatible

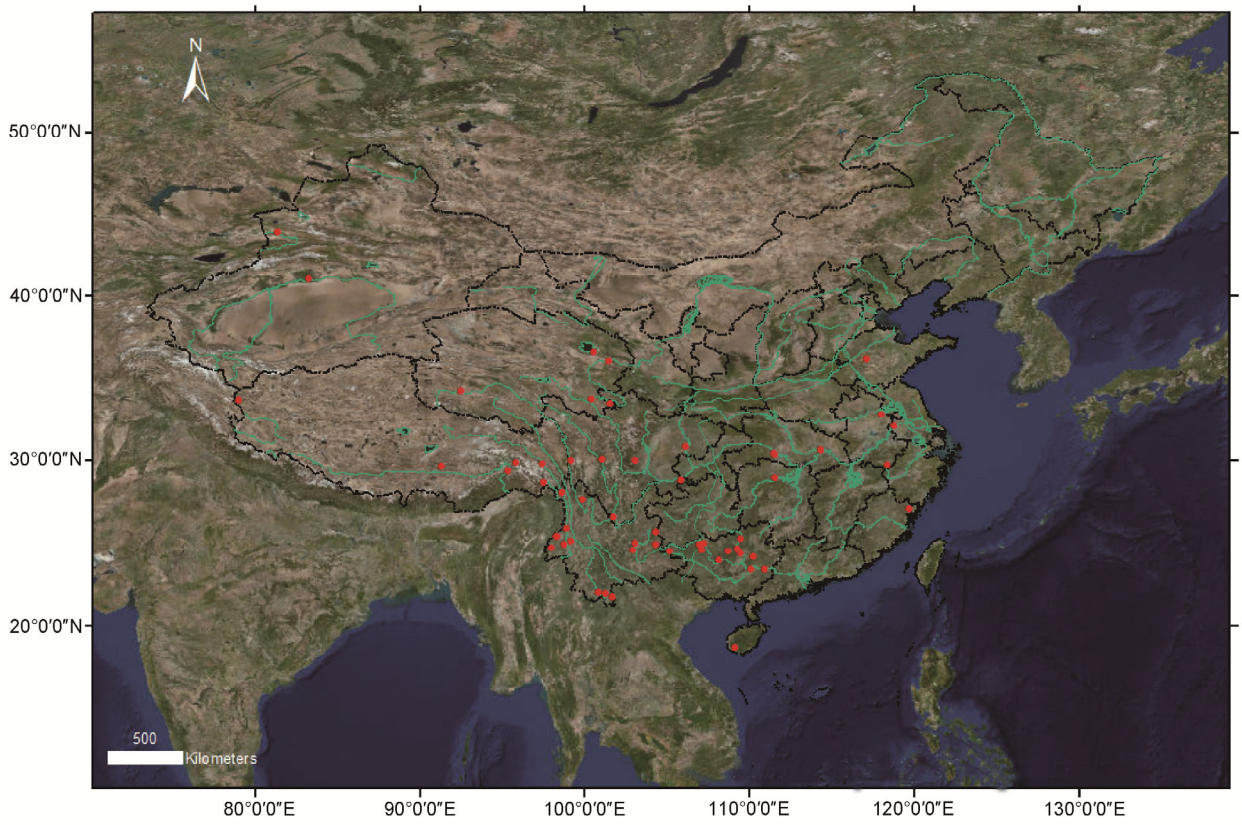

Figure 1 Map of locations of the Cyprininae ingroup taxa sampled in China in this study.

phylogenetic trees of the cyprininae fishes (Figure S1 in Supporting Information). As expected, the monophyly of the subfamily Cyprininae was strongly supported with bootstrap proportion of 99%–100% (in MP and ML analyses) and a posterior probability of 1.0 (in Bayesian analyses). Within the subfamily, the monophyly of labeonine fishes was strongly corroborated with high bootstrap support (⩾94%) and posterior probability (1.0), and the monophyly of the remaining cyprinine fishes (including cyprinine, barbine and schizothoracine) was supported with a posterior probability of 0.94 in the Bayesian analysis and a relatively moderate bootstrap proportion of 77% and 58% in the ML and MP analyses, respectively. As for the plateau polyploid cyprinids, two clades (node SCH and SPE) were also recovered with strong bootstrap proportion of 99%–100% (in MP and ML analyses) and a posterior probability of 1.0 (in Bayesian analyses). The clade SPE was a sister group to the *Scaphiodonichthys acanthopterus*+*Barbaus barbus*, and the clade SCH to the *Percocypris*, both with high bootstrap and posterior probability values (Figure S1 in Supporting Information).

State reconstructions for ploidy levels

The cladogram resulted from Bayesian analyses were used for state reconstructions for ploidy level of cyprinid fishes. Reconstructions showed that diploidy was the ancestral state in both the family Cyprinidae (node CYD, Figure 2) and the subfamily Cyprininae (node CYN, Figure 2), and switch from diploidy to tetraploidy was first found in the tribe barbine (node BAR, Figure 2). At least three independent switches to polyploidy and three reversals to diploidy were inferred within the tribe barbine, which possessed the most common ancestor of tetraploid.

Molecular dating

A molecular clock was rejected for the combined sequence data by the likelihood ratio test with likelihood scores of clock and non-clock model (χ^2=2279.686, df=148, P<0.05). Therefore, the relaxed molecular clock method was applied to estimate divergence time. The topology resulting from Bayesian analysis was used for dating phylogenetic events because of relatively well-resolved phylogenetic relationships. The subfamily Cyprininae began to diversify around 23.69 Mya with the 95% confidence interval of 23.16–24.33 Mya (node CYN in the Figure 2 and Table 1). Within the Cyprininae, the clade labeonine radiation (node LAB in the Figure 2 and Table 1) began around 21.65 Mya (the 95% credibility interval of 20.67–22.50 Mya), and the barbine (node BAR in the Figure 2 and Table 1) began to diversify around 23.21 Mya (the 95% credibility interval of 22.49–24.09 Mya).

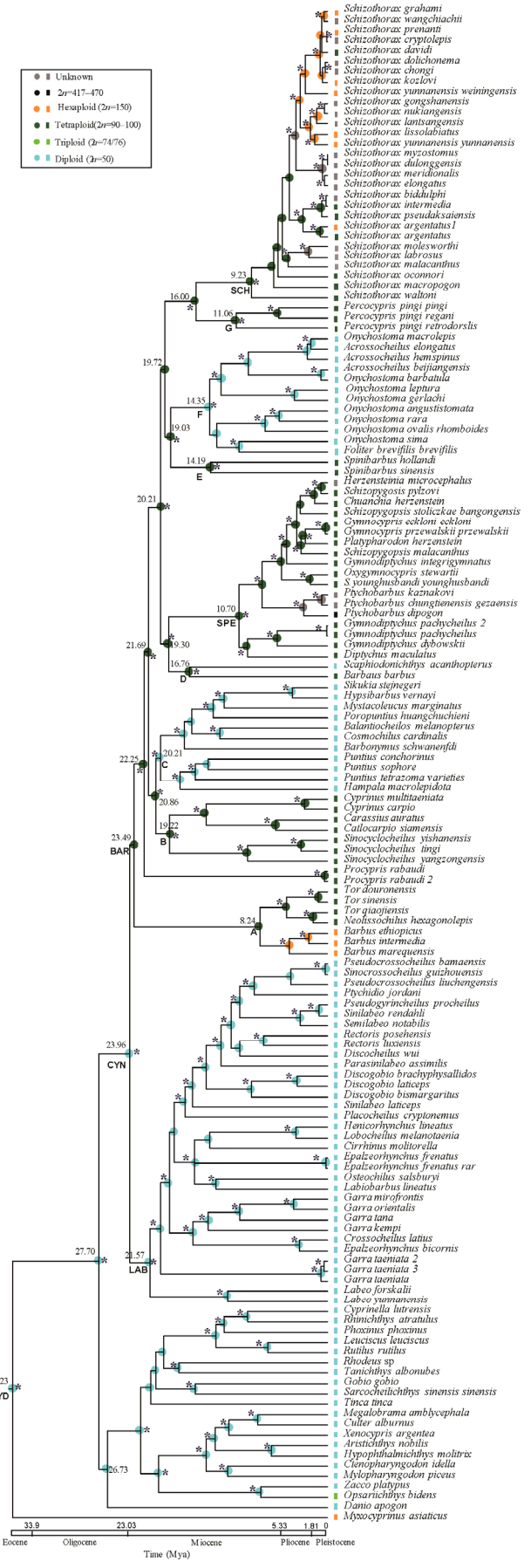

Figure 2 Chronogram of the Cyprininae fishes. Divergence date estimates were based on the tree topology resulting from the Bayesian analysis, and nodes with strong posterior probability supports ⩾0.95 were marked with asterisks (*). The numbers near the nodes represent the divergence dates (Mya) estimated, and node symbols for the key nodes are listed in Table 1 with mean divergence dates and 95% confidence intervals. The karyotypes of species analyzed and the ancestral states for ploidy levels reconstructed were marked with color bars and dots, respectively (Arai, 2011).

Lineage diversification

Overall, the P values for six tree balance statistics computed in SymmeTREE increased from I_C to B_1 (I_C=0.009718, M_Π^*=0.00095, M_Π=0.00645, M_Σ^*=0.004094, M_Σ=0.083688, B_1=0.37156) (Table S1 in Supporting Information), and the six tree statistics are known to be sensitive to the different nodal depth (listed from the shallowest to the deepest as above). The results suggested that lineage diversification rate variation was significantly concentrated at relatively shallow nodes within the Cyprininae tree. The delta statistics (Δ_1 and Δ_2) (Moore et al., 2004) and the Slowinski and Guyer statistic (Slowinski and Guyer, 1993) helped to locate a single shift point of diversification rate on the tree. In addition, the relative cladogenesis test detected rapid diversification rate shifts along 15 branches. These shift points on the tree were found only within the barbine clade (Figure 2 and Table 1) and confined to Miocene, Pliocene, and Early Pleistocene (23.0–0.9 Mya) with most of shifts (except those in the branch 1–3, 6, and 7) recovered in the last 12 Mya (from the Late Miocene to Pleistocene). These patterns were relatively robust because analyses of 100 randomly sampled trees based on Bayesian analysis detected diversification rate shifts in the branches of comparable age and placement on the tree.

A semilogarithmic plot of lineages through time (LTT) showed a significant deviation from a simulated plot obtained under a birth-death model in which incomplete taxon sampling and a constant deviation rate were considered. The pattern of the empirical LTT plot witnessed a decrease in diversification rate taking place from the Middle Miocene and a trend toward increased diversification rate beginning from the Middle Pleistocene. The birth-death likelihood (BDL) analysis chose pureBirth as the best constant rate model, which was therefore used as the null to test hypotheses with rate variable models. Results of BDL analysis selected yule3rate as the best rate variable model ($\Delta AICrc$=14.62078, P<0.05) (Table S2 in Supporting Information) and thereby rejected the null hypothesis of rate constancy. The BDL analysis corroborated evidence from the deviation of the empirical LTT plot from the simulated curve with 95% confidence intervals. According to the scenario suggested by the yule3rate model, the net diversification rate experienced two dramatic shifts in the evolutionary history of the Cyprininae diversification, which took place 16.0 and 0.91 Mya, respectively. The rate decreased 16 Mya from 0.28 to 0.09 speciation events per million years and

Table 1 Divergence time estimates of the key nodes in the Cyprininae phylogeny of Figure 2 and 95% confidence interval resulting from the lengthy bootstrap analysis.

Clade	Node	Age estimates (Myr ago)	
		Mean±SD	95% interval
Cyprinidae	CYD	38.22±0.92	33.84–38.42
Subfamily Cyprininae	CYN	23.96±0.31	23.16–24.33
Barbine clade, the subfamily Cyprininae excluding the labeonine fishes	BAR	23.49±0.33	22.49–24.09
Labeonine clade	LAB	21.57±0.44	20.67–22.50
Schizothorax	SCH	9.23±0.35	8.96–9.83
Schizothoracine fishes excluding the genus Schizothorax	SPE	10.70±0.54	9.64–11.78

shift again around 0.91 Mya with the rate increasing to 0.17 speciation events per million years.

DISCUSSION

The phylogenetic results presented here provide molecular evidence to explore the effects of Tibetan Plateau on the evolution of biogeographic patterns and ploidy levels of the cyprinine fishes. In total, our date estimation placed the diversification of polyploid cyprinids (including characteristically Tibetan cyprinids) on geologic time range corresponding well with the major phase of uplift of the Tibetan Plateau since the early Miocene (Guo et al., 2002; Harrison et al., 1992; Molnar, 2005; Shi et al., 1999; Zheng et al., 2013; Zheng et al., 2000).

Biogeographic patterns and ploidy levels in the Cyprininae

Our resolved phylogeny of the Cyprininae is well supported and confirms monophyly of the Cyprininae. Within the Cyprininae, the present analyses provide robust evidence for sister group relationship of the currently recognized labeonine to the clade containing the cyprinine, barbine, and schizothoracine species.

The strongly recovered clade LAB, sister to the rest of the subfamily Cyprininae, consists of only diploidy species, and origin of these diploid labeonine fishes is dated to the Late Oligocene (~24 Mya, Figure 2). The diploid labeonins are restricted to habitats of warm water and relatively low altitude, with widespread but disjunct distribution in Africa and Asia, predominantly in South East Asia. Such specialized distributions to swift currents have been explained by affinity with the greatest diversity in lip morphology and its associated structures that supposedly provided great ability of algae scrape for these species.

The ploidy level of the clade BAR is the most sophisticated in the family Cyprinidae, and several different ploidy levels within this group have been recovered. This clade predominantly consists of polyploid cyprinids, with an exception of at least two diploid clades (Clade C and F, Figure 2). Cladogenesis of the polyploid cyprinids began since about 23.5 Mya, just after the split time between the clade LAB and the clade BAR, concomitant with other lineages endemic to the Tibetan Plateau (Guo et al., 2005; Liu et al., 2006). The initial speciation within the polyploid clade led to origin of a polyploid clade comprising both tetraploid and hexaploid species (Clade A, Figure 2). The distant distribution between the tetraploids and hexaploids is remarkable in the clade A, with the distribution of the tetraploids in South and South East Asia and that of the hexaploids widely in Africa. Sister group of the tetraploid clade B to the diploid clade C was alsp supported by our phylogenetic reconstruction. The clade B comprises the tetraploid *Sinocyclocheilus*, *Cyprinus*, *Carassius* and *Catlocarpio*, while the clade C a group of diploid minnows. Distribution of the tetraploids in the clade B largely overlaps with that of the diploids in the clade C in the ranges from South China to Maeklong, Mekong and Chao Phraya basins in South East Asia.

Morphologically, the schizothoracins was recognized as a subfamily consisting of three groups, such as primitive group, specialized group, and highly specialized group (Cao et al., 1981). However, the morphological grouping of the schizothoracins had been challenged by recent molecular evidences (Li et al., 2008; Yang et al., 2015a; Yonezawa et al., 2014). The present phylogeny revealed two independent origins of these Tibetan Plateau endemic polyploid cyprinids, migrating independently of each other to the Tibetan Plateau and adapted to high altitude (Yonezawa et al., 2014). One schizothoracin clade SCH comprised taxa from the morphologically primitive group, and another schizothoracine clade SPE from morphologically specialized and highly specialized groups (Cao et al., 1981). Origins of the Tibetan Plateau clade SCH and SPE occurred in the Early Miocene, with crown diversification of these schizothoracin clades beginning from the Late Miocene to the Pleistocene.

The schizothoracin clade SPE represents a polyploid cyprinid group comprising taxa nearly all of that are tetraploid. However, our phylogeny supports a close relationship between the clade SPE and the clade D, consisting of the tetraploid *Barbus barbus* and the diploid *Scaphiodonichthys*. Divergence of the clade SPE from the clade *Barbus*+*Scaphiodonichthys* was dated to 19.30 Mya, diversification within the SPE began from the Late Miocene (Figure 2). Biogeographically, the clade SPE has a semicircular range around the Takla Makan encompassing the Tien Shan, Hindu Kush and Himalaya (Howes, 1991); while *Barbus barbus* a wide Europe distribution with the eastern limit marked by the Urals and the southern by the Tien

Shan, and the *Scaphiodonichthys* a narrow distribution of Mekong and Chao Phraya basins. The close relationship between the clade SPE and the *Barbus barbus*+*Scaphiodonichthys* suggested that their most recent common ancestor perhaps has a broad distribution in Eurasia.

The schizothoracin clade SCH comprises the *Schizothorax* taxa of high ploidy levels (tetraploids and hexaploids species), and ranges from the Tien Shan and Hindu Kush to Himalaya surrounding the Takla Makan (Howes, 1991). The *Schizothorax* clade is recovered as sister group to the tetraploid *Percocypris* (clade G, Figure 2), which currently occurs in areas including Jinshajiang, Salween and Mekong basins. Further, the clade SCH+G is sister to a monophyly consisting of a diploid *Onychostoma*+*Acrossocheilus* calde F and a tetraploid *Spinibarbus* clade E. These two clades, E and F, lying in Laos, Viet Nam and South-East China, have a restricted distribution in South East Asia greatly sympatric with that of the *Percocypris*. Common origin of the *Schizothorax* and the *Percocypris* and then split between them were estimated to take place in the Early Miocene (19.7 and 16.0 Mya, respectively, Figure 2). Consequently, the extant *Schizothorax* taxa are restricted to the high altitude drainages of the Tibetan Plateau and its adjacent regions. Based on the present phylogeny and biogeographic patterns, it can be hypothesized that these *Schizothorax* species may represent remnants of a more broadly South East Asia distributed tetraploid ancestor that became fragmented during the Late Miocene uplift of the Tibetan Plateau and survived in high altitude areas only.

Evolution of ploidy levels in the Cyprininae

Several different ploidy levels are represented in some clades within the subfamily Cyprininae, and this gives an opportunity to trace the evolutionary history of the polyploid species. The phylogenetic analyses performed for the current study indicated that the subfamily Cyprininae was thought to be diploid in origin and the barbine to be tetraploid in origin (Clade BAR, Figure 2). Within the barbine, species included in the tetraploid group occur in the genera *Barbus*, *Carassius*, *Cyprinus*, *Neolissocheilus*, *Percocypris*, *Pseudobarbus*, *Schizothorax*, *Sinocyclocheilus*, *Spinibarbus* and *Tor* etc (Arai, 2011). Tetraploid cpyrinids can arise from the diploid ancestor through autotetraploidization with spermatozoon and ovum each containing a diploid complement of chromosomes (Oellermann and Skelton, 1990). The advantageous traits of polyploidy for cyprinids fish might be summed up as large body size, fast growth rate, long life and great ecological adaptability (Schultz, 1980; Uyeno and Smith, 1972). For example, the hexaploid *Barbus* species in Africa (Clade A, Figure 2) are all found to be relatively large, long-lived and ecologically flexible species (Oellermann and Skelton, 1990). Despite of the advantages of polyploidy in evolution, reversal switches, by reducing the number of chromosomes from the tetraploid to the diploid level, were also inferred within the barbine.

Polyploidization has obviously been of great importance in the evolution of species of the schizothoracin clades, as is the case for all groups of these Tibetan Plateau endemic cyprinids. To date, all the schizothoracin species analyzed cytologically have revealed a high number of chromosomes ranging from tetraploid to hexaploid (even $2n$=446). The schizothoracin clades SCH and SPE were revealed to be tetreploid origins, and at least two independent switches to hexaploidy were inferred in the *Schizothorax*. Besides the tetraploid species in the genus *Schizothorax*, those hexaploidic species are of great interest in studies of evolution and survival of the Tibetan Plateau endemic cyprinids. Although mechanism of origin of hexaploidy in the *Schizothorax* is still poorly understood, the allohexaploidy was thought to be the most plausible ways in which hexaploidy may develop (Oellermann and Skelton, 1990). The role of polyploidy in evolution is very important because it helps fishes to escape natural selective pressures by providing redundant gene loci (Becak et al., 1966) and therefore easily accumulating mutations (Wolf et al., 1969). Polyploidization has been thought as a key factor that enables or even drives diversification in the schizothoracin groups. Therefore, we may hypothesize that the higher success of the polyploidy *Schizothorax* species at recolonizing high altitude habitats freed by uplift of the Tibetan Plateau might be explained in terms of selection for cold temperature and hypoxia. This hypothesis can be evidenced with viable tetraploid produced by giving the fertilized eggs a temperature shock (a sharp decrease in temperature) (Don and Avtalion, 1988). The other evidence may come from the Tibetan cyprinid, *Gymnodiptychus pachycheilus*. Genes in *G. pachycheilus* found with signature of rapid evolution and positive selection were functionnally associated with energy metabolism and hypoxia (Yang et al., 2015b).

Patterns of diversifications of the schizothoracins

The temporal BDL analysis and the LTT plot indicated an Early Miocene adaptation radiation of the Cyprininae fishes from ~23 to 16 Mya (shifts 1, 2, and 6; Figure 3), with the shifts 2 and 6 involved in diversification of stem groups of the Tibetan characteristic cyprinid lineages. The proposed Early Miocene adaptive radiation of the Cyprininae closely coincided with uplift of the Tibetan Plateau from the Late Oligocene to the Early Miocene (~25–17 Mya), because this period was marked as an important transition in the uplift of the South Tibet and the Himalaya, and the Himalayan-Tibetan region might have reached its elevation of about 2,000 m during this period (Shi et al., 1999). Furthermore, there is a growing body of evidence suggesting that the South Tibetan plateau may have started its fast uplift since 20 Mya and might have experienced a pulse of rapid uplift around 20–15 Mya (Copeland et al., 1987; Harrison et al., 1992). Massive alterations of environment associated with the Late Oligocene-Early Miocene uplift of the Tibetan Plateau ($\approx$25–17 Mya), including replacement

of tropical/subtropical rain forest by semi-humid/ semi-arid forest in the Southern Tibet, might be a result of the climatic change from the warm, dry climate of the Oligocene to moderately cool and wet conditions at the beginning of the early Miocene, followed by progressive cooling and drying (Wu et al., 2008). The interpretation of relating the Oligocene-Miocene origin (24–19 Mya) of the sisorid catfishes typical of the Tibetan Plateau to the Tibetan uplift contributed to the hypothesis that speciation of the Tibetan lineages might have been promoted by the significant environmental and habitat heterogeneity associated with the Early Miocene uplift of the Himalayas (Guo et al., 2005; Zhou et al., 2016). Therefore, we proposed that the Early Miocene diversification of stem groups of the schizothoracine fishes agree with this hypothesis and the adaptive radiation can be regarded as connections of environment and habitat response to the Early Miocene uplift of the Himalaya and Southern Tibet.

A slowdown of Cyprininae diversification, suggesting a decrease in origination rate and/or an increase in extinction rate, was evidenced by the present analyses during the Middle Miocene to the Early Pleistocene (about 16–1 Mya), a time period broadly coincident with the major phase of the Neogene Tibetan uplift. The closely nested diversification rate shifts during the Late Miocene to Early Pleistocene (~12–1 Mya) (shifts 4, 5, and 7–15) occurred only in two characteristically Tibetan lineages of polyploidy cyprinids, with one (shifts 7–15) composed entirely of the cleft breast cyprinids in the genus *Schizothorax* and the other (shifts 4 and 5) the highly specialized schizothoracine fishes except the *Schizothorax* (clade SCH and SPE, respectively, Figure 3). Although the southern Tibetan Plateau underwent its main stage of fast uplift in the Early Miocene and the elevation probably remained unchanged over the past 15 million years (Harris, 2006; Spicer et al., 2003), further significant uplifts (with possible intervening deformation) of the east and north of Tibet took place within recent 15 million years (Lu et al., 2004; Ritts et al., 2008). Geological evidence suggested that rapid uplift might take place in Eastern Tibet since about 15 Mya because an cessation of rapid Pacific trench migration during the Early to Middle Miocene (~20–15 Mya) probably contributed to the onset of rapid surface uplift and crustal thickening in Eastern Tibet (Royden et al., 2008). Additionally, a switch of the tectonic regime of the Tibetan Plateau from north-south convergence to east-west extension starting before ~14 Mya (Coleman and Hodges, 1995) was also the major phase of the Neogene uplift of the Tibetan Plateau. Although the time when the Tibet Plateau first reached its present elevation is still contentious (Guo et al., 2002; Harrison et al., 1992; Li and Fang, 1999; Li, 1996; Zheng et al., 2000), the Neogene did witness the Tibetan plateau attaining its maximum height. Therefore, the Neogene tectonic processes of the Tibet (beginning ~15 Mya) was necessarily linked with global climatic changes and even played an important role in shaping variation of climate and environments in East Asia. The Neogene variation of climate and environments associated with the Tibetan uplift includes strong oscillations between aridity and humid conditions (Fan et al., 2007), changes in vegetation (Cerling et al., 1997; Ma et al., 1998), open and close of paleodrainages (Fan et al., 2007), onset and evolution of Asian monsoons (An et al., 2001; Guo et al., 2002). Therefore, we hypothesize that variations of ecosystems associated with the severe tectonic process of the Tibetan Plateau ~16.0–1.0 Mya might have resulted in retreat of the Tibet endemic cyprinids to high altitudes and these schizothoracine fishes achieved their stabilization and establishment in new habitats because of the advantages of their polyploid condition in the process of extending the distribution. The Neogene shifts (beginning 16.0 Mya) closely coincided with crown diversification of the Tibetan Plateau endemic polyploidy cyprinids (clade SCH and SPE, Figure 3), probably indicating a preservation of comparatively ancient diversity resulting from the Early Miocene adaptive radiation of the stem groups.

We observed a slight increase in diversification rate in the LTT plot since 0.91 Mya (Figure 3). This increase is closely coincident with the successive Kunlun-Huanghe movement at 1.1–0.6 Mya and Gonghe movement at 0.15 Mya, through which the Tibetan Plateau raised to its present height (Li and Fang, 1999). The cladogenetic events within the *Schizothorax* fishes (Clade SCH, Figure 3) contributed more to the diversification rate increase from the point (0.91 Mya) forward to the present. Therefore, the recent diversification was influenced by the Quaternary climatic and tectonic events in the Tibetan Plateau and surroundings, such as the intensification of Asian monsoons, cryosphere of the Tibetan Plateau, and development of the large-scale Plateau glaciations. The relative stability of the Tibetan environments and the glaciations-induced climatic oscillations during the late Early Pleistocene to the present may have helped the speciation accumulation of the polyploidy cyprinids.

The reconstructed evolutionary history of diversification of the Tibetan polyploidy cyprinids within the Cyprininae indicated that crown diversification of the Tibetan endemic cyprinids (schizothoracine fishes) may have occurred relatively early (in the early Middle Miocene), with a recent diversification in response to the Quaternary climatic and tectonic events, such as Pleistocene glaciation and Pleistocene uplift of the Tibetan Plateau. In general, temporal patterns of inconstant diversification rate seem to be consistent with cradle models (recent and rapid diversification) or museum models (slow accumulation of diversity over time and/or preservation of ancient diversity) or both. Therefore, our results lead to a hypothesis that the present schizothraocine fishes diversity maybe resulted from both comparatively ancient adaptive radiation and recent rapid diversification. The polyploid condition of the schizothoracine fishes may be of great help in the preservation process of the *Schizothorax* diversity in the Quaternary era. Furthermore,

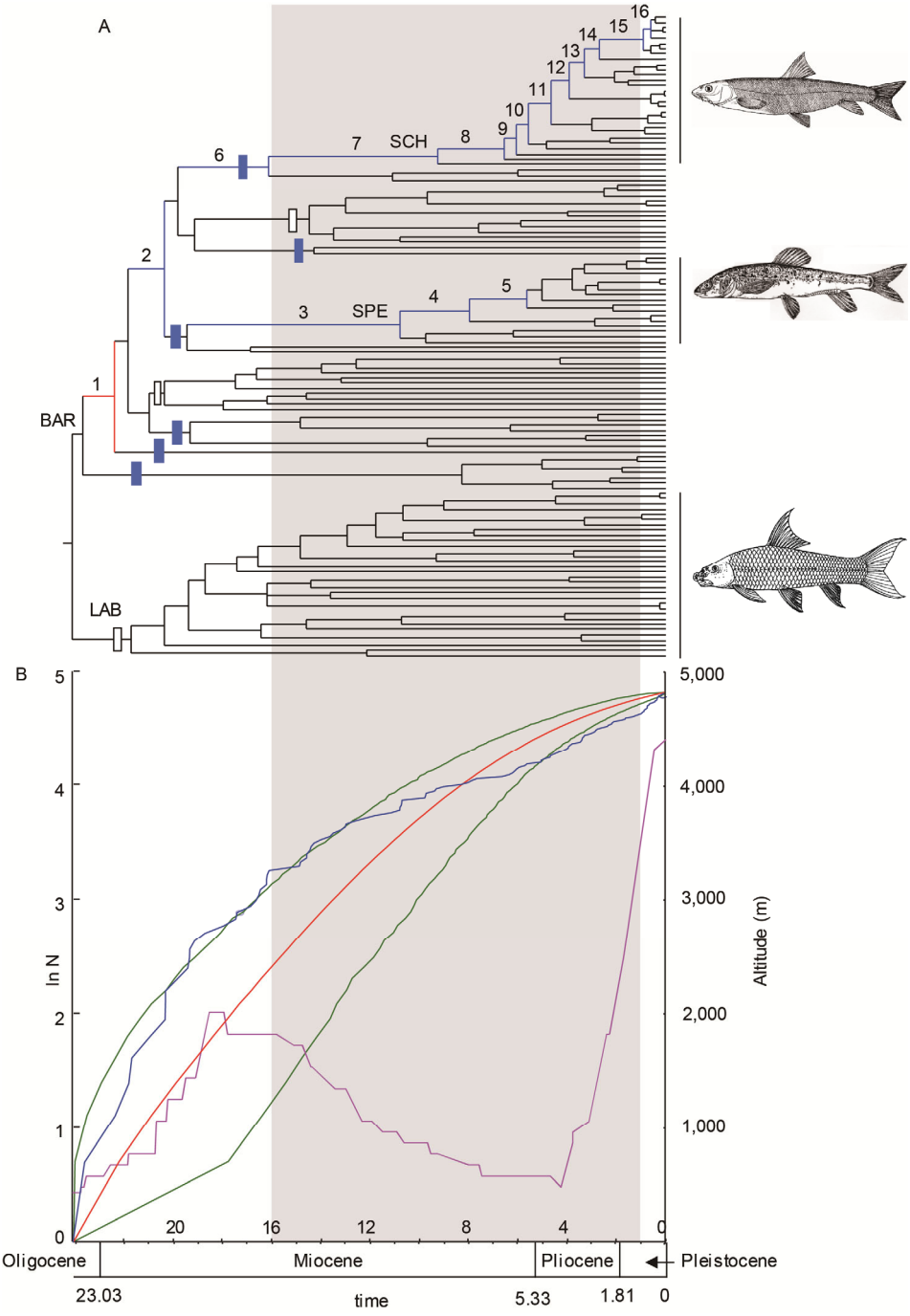

Figure 3 Timing and tempo in the evolutionary radiations of cyprininae fishes. A, The PL ingroup chronogram. The Chronogram is the Bayesian tree estimated using PL method with all non-Cyprininae taxa and duplicate species culled from the chronogram. The significant diversification rate shifts detected by different "shift" statistics are marked with Arabic numerals and colors: shift 1 (red) is supported by the Δ_1, Δ_2, and Slowinski and Guyer statistics; the other shifts (shifts 2–16, blue) are supported by the relative cladogenesis test. The blue rectangle bars on the branches indicate clades subtending polyploid cyprinine fishes, and the white bars those diploid cyprinine fishes. Three representative specimens are shown as examples near their respective positions in the chronogram. B, Semilogarithmic plot of LTT for the Cyprininae (LTT plot; blue) superimposed on the approximately hypothesized altitudes of the Tibetan Plateau during the late Cenozoic Era (pink dashed line, adapted from (Li and Fang, 1999)) and simulated LTT plot (red) with 95% confidence intervals (green) with a constant death-birth rate of 0.5. Upturns or downturns in the empirical LTT plot reflect changes in diversification rate. Prolonged period of decreased diversification rate is highlighted by shaded area.

climatic, and biotic events associated with the Tibetan Schizothoracine may serve as a good model to provide evidence for the hypothesis that major tectonic, the Tibetan Plateau may have most influenced on diversification at the proper geographic/taxonomic (e.g., Tibetan and its adjacently regional or characteristically lineagespecific) and

temporal (e.g., Neogene and Quaternary) scales.

MATERIALS AND METHODS

Sample collection

Ingroup samples in this study included 123 species of the subfamily Cyprininae, distributed in 46 genera and 20 non-Cyprininae cyprinid species. Based on the consensus that Cypriniformes is a monophyletic group, three cypriniforme species out Cyprinidae (*Myxocyprinus asiaticus*, *Gyrinocheilus* sp., and *Misgurnus* sp.) were selected as outgroup (Table 2, Figure 2). All tissues used for DNA extraction were preserved in 95% ethanol and corresponding specimens were deposited in the Freshwater Fish Museum of Institute of Hydrobiology of Chinese Academy of Sciences.

DNA sequence data collection and alignment

Field collections of muscle or fin tissues were fixed in 95% ethanol alcohol and kept at –20°C in the laboratory until the time of DNA extraction. Total genomic DNA was isolated from muscle or fin tissues using phenol/chloroform extraction procedure (Sambrook et al., 1989). The nuclear *RAG2* gene and the mitochondrial genes were amplified from total DNA extracts via polymerase chain reaction (PCR) using published and/or optimized primers (Table S3 in Supporting Information). Reaction mixtures contained approximately 100 ng of DNA template, 5 μL of 10× reaction buffer, 2 μL dNTPs (each 2.5 mmol L^{-1}), 2.0 U Taq polymerase, and 1 μL of each oligonucleotide primer, each at 10 μmol L^{-1} concentrations, in a final volume 50 μL. The PCR amplification profile included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation of 30 s at 94°C, annealing of 30 s at 45°C–56°C (annealing temperature depended on gene amplified), extension of 90 s at 72°C, and a final extension of 8 min at 72°C. Amplified DNA was fractionated by electrophoresis through 0.8% low-melting agarose gels, recovered from the gels, and purified using BioStar Glassmilk DNA purification Kit according to manufacturer's instructions. Nucleotide sequences of the *RAG2* gene and the mitochondrial *16S rRNA* gene were determined using purified PCR product. All sequences have been deposited in GenBank (Table 2).

For the protein-encoding genes, multiple alignments of sequences were performed using CLUSTAL X (Thompson et al., 1997). For the mitochondrial *16S rRNA* gene, sequences were initially aligned using CLUSTAL X, and the aligned sequences were finally viewed and further manually aligned based on secondary structural elements and conserved motifs, by comparing to existing models of the mitochondrial *16S rRNA* secondary structure for the cyprinid fishes (De Rijk et al., 2000; Gutell and Fox, 1988; Gutell et al., 1993). For the protein-coding genes, preliminary alignments were performed using CLUSTAL X (Thompson et al., 1997), then the computer-generated alignments were carefully adjusted manually by comparison with amino acid sequences implemented in MacClade 4.0 (Maddison and Maddison, 2000).

Phylogenetic analyses and evolution of ploidy levels

The combined data of DNA sequences was then used for the following phylogenetic analyses. Maximum parsimony (MP) analyses were conducted using PAUP*4.0b10. Equally weighted parsimony heuristic tree search was performed using tree bisection-reconnection (TBR) branch swapping and 1,000 random sequence addition replicates. Robustness of the nodes recovered was evaluated with a nonparametric bootstrap analysis (Felsenstein, 1985) of 1,000 pseudoreplicates and decay indices were generated with the program Autodecay (Eriksson, 2001). The ML analyses were performed using the program RAxML (Stamatakis, 2006). We searched for the ML topology with the highest likelihood during the 2,000 searches, using bootstrap analyses with 500 replicate to measure support for the recovered clades. The GTRGAMMAIG model was employed in the ML analyses.

Bayesian phylogenetic analyses were performed using the software MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Best-fit model of nucleotide substitution for the combined data was selected with the hierarchical likelihood ratio test using the program Modeltest3.7 (Posada and Crandall, 1998). Metropolis-coupled MCMC analyses were run for 2×10^7 generations, with one in every 1,000 generations being sampled. In order to identify and discard the generations prior to stationarity, we used the program Tracer (version 1.4) to plot log-likelihood scores, tree lengths, and all model parameter values against generation number. These graphical inspection analyses can help us evaluate "burn-in" generations. Our chain convergence diagnoses suggested that chain convergence generally occurred within the first 2 million generations, and we followed a conservative approach by discarding the first 10 million generations (10,000 sampled trees) as burn-in and using the remaining 1,000,000 generations (10,000 sampled trees) in all subsequent analysis. The 50% majority-rule consensus trees were generated with mean branch-length estimates, posterior probability values for each node, credible sets of trees, and parameter estimates.

To trace evolution of ploidy levels within the cyprinine fishes, we map the states of fish karyotypes onto the cladogram from the above phylogenetic analyses. The ancestral states for ploidy levels were reconstructed by using the function implemented in Mesquite (Maddison and Maddison, 2015), with the parameter of trace characters over trees, and ancestral state reconstruction method of parsimony ancestral states.

Divergence time estimation

The rate constancy along the evolutionary lineages across

Table 2 Samples of cyprinine ingroup and outgroup taxa collected in this study, and coordinate of locality also provided for taxa sampled in China within the brackets

Subfamily	Species	Locality (latitude, longitude)	Genes Sequences				
			RAG 2	*16S rRNA*	*CoI*	*ND4*	*Cyt b*
Outgroup	*Gyrinocheilus* sp.	Genbank	AY804074*	A2164*	A2164*	A2164*	A2164*
	Misgurnus sp.	Genbank	AY804103*	A2171*	A2171*	A2171*	A2171*
	Myxocyprinus asiaticus	Wuhan, Hubei Prov. (30.598, 114.312)	DQ367043	DQ845896	AY526869*	AY526869*	AY986503*
	Aristichthys nobilis	Wuhan, Hubei Prov. (30.598, 114.312)	DQ367038	GQ406281	HQ236000	HQ235871	AF051855*
	Ctenopharyngodon idella	Hengxian, Guangxi Prov. (22.686, 109.268)	DQ366996	GQ406277	HQ236004	HQ235869	AF051860*
	Culter alburnus	Taoyuan, Hunan Prov. (28.909, 111.495)	DQ367004	GQ406293	GU190362*	GU190362*	AP009060*
	Cyprinella lutrensis	GN531	DQ367019	GQ406267	A0206*	A0206*	A0206*
	Danio apogon	Genbank	U71094*	A4175*	A4175*	A4175*	A4175*
	Gobio gobio	France	DQ367015	GQ406305	A9596*	A9596*	AY426592*
	Hypophthalmichthys molitrix	Chenxi, Hunan Prov. (28.013, 110.190)	DQ367002	GQ406282	HQ236001	HQ235870	AF051866*
	Leuciscus leuciscus	France	DQ367007	GQ406268	–	–	AY509823*
	Megalobrama amblycephala	Wuhan, Hubei Prov. (30.598, 114.312)	DQ367025	GQ406294	EU434747*	EU434747*	AF051867*
	Mylopharyngodon piceus	Taoyuan, Hunan Prov. (28.909, 111.495)	DQ367011	GQ406278	HQ236003	HQ235872	AF051870*
	Opsariichthys bidens	Taoyuan, Hunan Prov. (28.909, 111.495)	DQ367014	GQ406289	DQ367044*	DQ367044*	DQ367044*
	Phoxinus phoxinus	Europe	DQ367022	GQ406269	–	–	Y10448*
	Rhinichthys atratulus	GN529	DQ367018	GQ406273	EU525120*	–	AF452078*
	Rhodeus sp.	Xilin, Guangxi Prov. (24.496, 105.100)	DQ367031	GQ406311	DQ026430*	DQ026430*	DQ026430*
	Rutilus rutilus	France	DQ367003	GQ406271	–	–	AF095610*
	Sarcocheilichthys sinensis sinensis	Hejiang, Sichuan Prov. (28.818, 105.838)	DQ367026	GQ406308	A4124*	A4124*	AY952983*
	Tanichthys albonubes	Aquarium	DQ367023	GQ406291	–	–	EF151121*
	Tinca tinca	Europe	DQ367029	GQ406280	A8686*	A8686*	Y10451*
	Xenocypris argentea	Taoyuan, Hunan Prov. (28.909, 111.495)	DQ367024	GQ406285	AP009059*	AP009059*	AP009059*
	Zacco platypus	Jinxiu, Guangxi Prov. (24.139, 110.195)	DQ367010	GQ406292	EF452896*	EF452825*	AY245048*
Labeoninae	*Cirrhinus molitorella*	Tengxian, Guangxi Prov. (23.381, 110.921)	DQ366959	DQ845883	HQ235977	HQ235791	AY463098*
	Crossocheilus latius 2	Tengchong, Yunan Prov. (25.380, 98.210)	DQ366982	DQ845882	–	–	–
	Discocheilus wui	Fengshan, Guangxi Prov. (24.553, 107.049)	HQ235874	HQ235699	–	HQ235797	HQ235747
	Discogobio bismargaritus	Liuzhou, Guangxi Prov. (24.332, 109.422)	DQ366947	DQ845890	–	–	GQ406318
	Discogobio brachyphysallidos	Jinxiu, Guangxi Prov. (24.139, 110.195)	DQ366958	DQ845901	–	–	GQ406319
	Discogobio laticeps	Tian'e, Guangxi Prov. (25.005, 107.180)	DQ366949	DQ845889	–	HQ235795	GQ406320
	Epalzeorhynchus bicornis	Baoshan, Yunnan Prov. (25.118, 99.168)	HQ235875	DQ845919	–	HQ235802	HQ235748
	Epalzeorhynchus frenatus	Aquarium	HQ235876	–	–	HQ235803	–
	Epalzeorhynchus frenatus rar	Aquarium	DQ366943	DQ845905	–	HQ235778	GQ406321
	Garra kempi	Chayu, Xizang Prov. (28.668, 97.473)	DQ366968	DQ845885	HQ235946	–	–
	Garra mirofrontis	Tengchong, Yunnan Prov.	DQ366934	HQ235700	–	HQ235793	HQ235749
	Garra orientalis	Ledong, Hainan Prov. (18.659, 109.063)	DQ366957	DQ845884	–	–	GQ406322
	Garra taeniata 2	Jinghong, Yunnan Prov. (22.006, 100.778)	HQ235877	HQ235701	–	–	–
	Garra taeniata 3	Mengla, Yunnan Prov. (21.696, 101.655)	HQ235878	HQ235702	–	–	–

(*To be continued on the next page*)

(*Continued*)

Subfamily	Species	Locality (latitude, longitude)	Genes Sequences				
			RAG 2	*16S rRNA*	*CoI*	*ND4*	*Cyt b*
Labeoninae	*Garra taeniata*	Jinghong, Yunnan Prov. (22.006, 100.778)	DQ366953	GQ406262	–	–	–
	Garra tana	Ethiopia	HQ235879	HQ235703	HQ235945	HQ235792	HQ235750
	Henicorhynchus lineatus	Menglun, Yunnan Prov. (21.941, 101.256)	DQ366935	GQ406263	–	HQ235789	GQ406323
	Labeo forskalii	Ethiopia	HQ235880	HQ235704	–	–	HQ235751
	Labeo yunnanensis	Mengla, Yunnan Prov. (21.696, 101.655)	DQ366948	DQ845881	–	HQ235794	GQ406324
	Labiobarbus lineatus	Mengla, Yunnan Prov. (21.696, 101.655)	HQ235881	DQ845914	–	HQ235790	HQ235752
	Lobocheilus melanotaenia	Menglun, Yunnan Prov. (21.941, 101.256)	DQ366940	DQ845902	–	–	DQ464990*
	Osteochilus salsburyi	Rong'an, Guangxi Prov. (25.230, 109.404)	DQ366971	DQ845892	–	HQ235804	GQ406325
	Parasinilabeo assimilis	Rong'an, Guangxi Prov. (25.230, 109.404)	DQ366992	DQ845887	–	HQ235798	GQ406326
	Placocheilus cryptonemus	Liuku, Yunnan Prov. (25.850, 98.861)	HQ235882	DQ845915	–	–	HQ235753
	Pseudocrossocheilus bamaensis	Tian'e, Guangxi Prov. (25.005, 107.180)	DQ366993	DQ845895	–	HQ235799	GQ406327
	Pseudocrossocheilus liuchengensis	Liucheng, Guangxi Prov. (24.656, 109.251)	HQ235883	–	–	–	–
	Pseudogyrincheilus procheilus	Nanchong, Sichuan Prov. (30.843, 106.117)	HQ235884	HQ235705	–	HQ235800	HQ235754
	Ptychidio jordani	Tian'e, Guangxi Prov. (25.005, 107.180)	DQ366974	DQ845893	–	HQ235838	GQ406328
	Rectoris luxiensis	Taoyuan, Hunan Prov. (28.909, 111.495)	DQ366977	–	–	–	–
	Rectoris posehensis	Dou'an, Guangxi Prov. (23.937, 108.112)	DQ366975	DQ845891	–	HQ235796	GQ406329
	Semilabeo notabilis	Jinxiu, Guangxi Prov. (24.139, 110.195)	DQ366983	DQ845886	–	HQ235801	GQ406330
	Sinilabeo laticeps	Mengla, Yunnan Prov. (21.696, 101.655)	HQ235885	DQ845904	–	HQ235779	HQ235755
	Sinilabeo rendahli	Yidu, Hubei Prov. (30.384, 111.4570)	DQ366932	GQ406264	–	–	–
	Sinocrossocheilus guizhouensis	Buliuhe, Guangxi Prov. (24.914, 106.944)	HQ235886	–	–	HQ235777	HQ235756
Cyprininae	*Carassius auratus*	Wuhan, Hubei Prov. (30.598, 114.312)	DQ366941	A6953*	A6953*	A6953*	A6953*
	Catlocarpio siamensis	Aquarium	HQ235887	HQ235706	–	–	HQ235757
	Cyprinus carpio	Tian'e, Guangxi Prov. (25.005, 107.180)	DQ366994	010*	010*	010*	010*
	Cyprinus multitaeniata	Guiping, Guangxi Prov. (23.400, 110.086)	DQ366939	DQ845845	–	HQ235776	HQ235758
	Procypris rabaudi	Hejiang, Sichuan Prov. (28.818, 105.838)	DQ366969	DQ845846	HQ235974	HQ235809	GQ406317
	Procypris rabaudi 2	Zhicheng, Hubei Prov. (30.304, 111.507)	HQ235888	HQ235707	HQ235975	HQ235811	HQ235759
Barbinae	*Acrossocheilus beijiangensis*	Rong'an, Guangxi Prov. (25.230, 109.404)	DQ366967	DQ845869	–	HQ235847	HQ235760
	Acrossocheilus elongatus	Rong'an, Guangxi Prov. (25.230, 109.404)	DQ366979	GQ406254	–	–	–
	Acrossocheilus hem/spinus	Rong'an, Guangxi Prov. (25.230, 109.404)	DQ366986	DQ845867	–	HQ235848	GQ406312
	Balantiocheilos melanopterus	Aquarium	DQ366933	GQ406255	–	–	–
	Barbonymus schwanenfdi	Aquarium	DQ366961	DQ845906	–	HQ235805	AF180823*
	Barbus barbus	France	DQ366990	DQ845879	HQ235999	–	A8965*
	Barbus ethiopicus	Ethiopia	HQ235889	HQ235708	HQ235947	HQ235781	AF180828*
	Barbus intermedia	Ethiopia	HQ235890	HQ235709	HQ235948	HQ235780	AF145948*
	Barbus marequensis	South Africa	HQ235891	–	HQ236007	–	AF180830*
	Cosmochilus cardinalis	Mengla, Yunnan Prov. (21.696, 101.655)	HQ235892	HQ235710	–	–	HQ235761
	Foliter brevifilis brevifilis	Tian'e, Guangxi Prov. (25.005, 107.180)	HQ235893	HQ235711	HQ236008	HQ235846	F1161*
	Hampala macrolepidota	Mengla, Yunnan Prov. (21.696, 101.655)	DQ366965	DQ845863	HQ236005	–	DQ464974*
	Hypsibarbus vernayi	Mengla, Yunnan Prov. (21.696, 101.655)	DQ366987	DQ845870	–	HQ235785	GQ406313
	Mystacoleucus marginatus	Mengla, Yunnan Prov. (21.696, 101.655)	HQ235894	HQ235712	–	HQ235806	HQ235762

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(*Continued*)

Subfamily	Species	Locality (latitude, longitude)	Genes Sequences				
			RAG 2	*16S rRNA*	*CoI*	*ND4*	*Cyt b*
Barbinae	*Neolissochilus hexagonolepis*	Motuo, Xizang Prov. (29.331, 95.340)	HQ235895	HQ235713	–	–	AY463516*
	Onychostoma angustistomata	Panzhihua, Sichuan Prov. (26.588, 101.725)	HQ235896	HQ235714	–	–	HQ235763
	Onychostoma barbatula	Fu'an, Fujian Prov. (27.094, 119.654)	DQ366964	–	–	–	–
	Onychostoma gerlachi	Jinghong, Yunnan Prov. (22.006, 100.778)	DQ366963	DQ845862	–	HQ235845	GQ406314
	Onychostoma leptura	Xilin, Guangxi Prov. (24.496, 105.100)	DQ366955	GQ406257	–	–	HM142578*
	Onychostoma macrolepis	Taian, Shandong Prov. (36.206, 117.095)	DQ366942	GQ406258	–	–	–
	Onychostoma ovalis rhomboides	Tian'e, Guangxi Prov. (25.005, 107.180)	DQ366988	–	–	HQ235826	–
	Onychostoma rara	Tian'e, Guangxi Prov. (25.005, 107.180)	DQ366984	HQ235715	–	HQ235844	HQ235764
	Onychostoma sima	Hejiang, Sichuan Prov. (28.818, 105.838)	DQ366991	DQ845861	–	HQ235812	HQ235765
	Percocypris pingi pingi	Hejiang, Sichuan Prov. (28.818, 105.838)	DQ366962	GQ406259	HQ235972	–	HQ235766
	Percocypris pingi regani	Fuyuan, Yunnan Prov. (25.680, 104.261)	HQ235897	HQ235716	HQ235971	HQ235832	HQ235767
	Percocypris pingi retrodorslis	Baoshan, Yunnan Prov. (25.118, 99.168)	HQ235898	HQ235717	HQ235973	HQ235833	HQ235768
	Poropuntius huangchuchieni	Mengla, Yunnan Prov. (21.696, 101.655)	DQ366952	HQ235718	–	HQ235807	HQ235769
	Puntius conchorinus	Aquarium,	GQ406253	DQ845880	–	HQ235783	AY004751*
	Puntius sophore	Ledong, Hainan Prov. (18.659, 109.063)	HQ235899	–	–	–	EU241461*
	Puntius tetrazoma varieties	Aquarium	DQ366938	EU287909*	–	HQ235782	EU287909*
	Scaphiodonichthys acanthopterus	Mengla, Yunnan Prov. (21.696, 101.655)	HQ235900	HQ235719	HQ236002	HQ235842	HQ235770
	Sikukia stejnegeri	Mengla, Yunnan Prov. (21.696, 101.655)	DQ366931	DQ845872	HQ236006	HQ235784	GQ406315
	Sinocyclocheilus tingi	Fuxianhu, Yunnan Prov. (24.542, 102.905)	DQ366978	DQ845866	–	AY854758*	AY854701*
	Sinocyclocheilus yangzongensis	Yangzonghai, Yunnan Prov. (24.959, 103.012)	HQ235902	DQ845926	–	AY854783*	AY854726*
	Sinocyclocheilus yishanensis	Yishan, Guangxi Prov. (24.491, 108.643)	HQ235903	DQ845908	–	–	A6445*
	Spinibarbus hollandi	Huangshan, Anhui Prov. (29.702, 118.322)	DQ366973	DQ845865	–	HQ235841	AY195628*
	Spinibarbus sinensis	Nanchong, Sichuan Prov. (30.843, 106.117)	HQ235904	DQ845864	–	HQ235808	HQ235771
	Tor douronensis	Menglun, Yunnan Prov. (21.941, 101.256)	DQ366945	DQ845877	–	HQ235786	DQ464986*
	Tor qiaojiensis	Yingjiang, Yunnan Prov. (24.711, 97.938)	DQ366970	DQ845873	–	–	GQ406316
	Tor sinensis	Mengla, Yunnan Prov. (21.696, 101.655)	DQ366936	DQ845876	–	HQ235788	F1164*
Schizothoracinae	*Chuanchia labiosa*	Guide, Qinghai Prov. (36.046, 101.440)	HQ235905	HQ235720	HQ235978	HQ235860	HQ235772
	Diptychus maculatus	Yili, Xijiang Prov. (43.898, 81.303)	HQ235906	HQ235721	HQ235994	HQ235827	AY463515*
	Gymnocypris eckloni eckloni	Huanghe, Qinghai Prov. (33.770, 100.391)	DQ366950	DQ845853	HQ235982	HQ235856	AY463522*
	Gymnocypris przewalskii przewalskii	Qinghai Lake, Qinghai Prov. (36.584, 100.502)	DQ366954	DQ845851	HQ235984	HQ235839	AY463523*
	Gymnodiptychus dybowskii	Yili, Xijiang Prov. (43.898, 81.303)	DQ366956	DQ845859	HQ235997	HQ235828	AY463513*
	Gymnodiptychus integrigymnatus	Mingguang, Yunnan Prov. (32.783, 117.996)	HQ235907	HQ235722	HQ235990	HQ235837	AY463527*
	Gymnodiptychus pachycheilus 2	Huanghe, Qinghai Prov. (33.770, 100.391)	HQ235908	–	HQ235996	HQ235862	–
	Gymnodiptychus pachycheilus	Huanghe, Qinghai Prov. (33.770, 100.391)	HQ235909	HQ235723	HQ235995	HQ235863	F1039*
	Herzensteinia microcephalus	Tuotuohe, Qinghai Prov. (34.223, 92.451)	HQ235910	HQ235724	HQ235980	HQ235861	DQ309354*
	Oxygymnocypris stewartii	Lasha, Xizang Prov. (29.665, 91.271)	HQ235911	DQ845918	HQ235988	HQ235836	DQ309358*
	Platypharodon extremus	Jiuzhi, Qinghai Prov. (33.435, 101.489)	HQ235912	HQ235725	HQ235985	HQ235858	F1023*
	Ptychobarbus chungtienensis gezaensis	Zhongdian, Yunnan Prov. (27.588, 99.798)	HQ235913	HQ235726	HQ235992	HQ235864	AY463506*
	Ptychobarbus dipogon	Lasha, Xizang Prov. (29.665, 91.271)	HQ235914	HQ235727	HQ235993	HQ235866	AY463510*
	Ptychobarbus kaznakovi	Zuogong, Xizang Prov. (29.812, 97.422)	HQ235915	DQ845916	HQ235991	HQ235865	AY463505*

(*To be continued on the next page*)

(*Continued*)

Subfamily	Species	Locality (latitude, longitude)	Genes Sequences				
			RAG 2	*16S rRNA*	*CoI*	*ND4*	*Cyt b*
Schizothoracinae	*Schizopygopsis malacanthus*	Yajiang, SichuanProv.	HQ235916	HQ235728	HQ235986	HQ235834	DQ309361*
	Schizopygopsis stoliczkae bangongensis	Bangonghu, Xizang Prov. (33.670, 78.745)	HQ235917	–	HQ235981	HQ235824	–
	S. younghusbandi younghusbandi	Bomi, Xizang Prov. (29.865, 95.774)	DQ366976	GQ406265	HQ235987	HQ235835	AY463501*
	Schizopygopsis pylzovi	Huanghe, Qinghai Prov. (33.770, 100.391)	HQ235918	DQ845856	HQ235979	HQ235859	DQ646897*
	Schizothorax argentatus	Yili, Xijiang Prov. (43.898, 81.303)	HQ235919	DQ845898	–	–	AF180861*
	Schizothorax argentatus 2	Yili, Xijiang Prov. (43.898, 81.303)	HQ235920	–	HQ235967	HQ235831	AY954269*
	Schizothorax biddulphi	Yili, Xijiang Prov. (43.898, 81.303)	HQ235921	–	HQ235966	HQ235829	F1464*
	Schizothorax chongi	Panzhihua, Sichuan Prov. (26.588, 101.725)	HQ235922	HQ235729	HQ235953	HQ235867	DQ126118*
	Schizothorax cryptolepis	Ya'an, Sichuan Prov. (30.021, 103.046)	HQ235923	HQ235730	HQ236010	HQ235815	HQ235773
	Schizothorax davidi	Ya'an, Sichuan Prov. (30.021, 103.046)	HQ235924	HQ235731	HQ236009	HQ235814	AY954257*
	Schizothorax dolichonema	Panzhihua, Sichuan Prov. (26.588, 101.725)	HQ235925	HQ235732	HQ235952	HQ235850	DQ126116*
	Schizothorax dulonggensis	Guyong, Yunnan Prov. (25.371, 98.318)	DQ366985	DQ845849	HQ235968	HQ235821	AY954284*
	Schizothorax elongatus	Tengchong, Yunan Prov. (25.380, 98.210)	HQ235926	HQ235733	HQ235970	HQ235820	–
	Schizothorax gongshanensis	Shipu, Yunnan Prov. (28.073, 98.582)	HQ235927	HQ235734	HQ235954	HQ235818	AY954279*
	Schizothorax grahami	Helongtan, Yunnan Prov. (33.770, 100.391)	HQ235928	HQ235735	HQ235944	HQ235843	HQ235774
	Schizothorax intermedia	Talimu, Xinjiang Prov. (41.061, 83.201)	HQ235929	HQ235736	HQ235965	HQ235825	AY954272*
	Schizothorax kozlovi	Batang, Sichuan Prov. (30.012, 99.117)	HQ235930	HQ235737	HQ235950	HQ235816	AY954256*
	Schizothorax labrosus	Chayu, Xizang Prov. (28.668, 97.473)	HQ235931	HQ235738	–	HQ235823	–
	Schizothorax lantsangensis	Wayao, Yunnan Prov. (24.924, 98.717)	HQ235932	DQ845911	HQ235956	HQ235853	DQ126126*
	Schizothorax lissolabiatus	Wayao, Yunnan Prov. (24.924, 98.717)	HQ235933	HQ235739	HQ235958	HQ235817	EU158042*
	Schizothorax macropogon	Lasha, Xizang Prov. (29.665, 91.271)	HQ235934	HQ235740	HQ235961	HQ235868	AY463517*
	Schizothorax malacanthus	Yajiang, Sichuan Prov. (30.038, 101.021)	HQ235935	HQ235741	HQ235962	HQ235822	AY954277*
	Schizothorax meridionalis	Yingjiang, Yunnan Prov. (24.711, 97.938)	DQ366989	DQ845847	–	–	AY954287*
	Schizothorax molesworthi	Chayu, Xizang Prov. (28.668, 97.473)	DQ366946	DQ845848	HQ235963	HQ235855	DQ126130*
	Schizothorax myzostomus	Guyong, Yunnan Prov. (25.371, 98.318)	DQ366960	DQ845850	HQ235969	–	GQ406331
	Schizothorax nukiangensis	Liuku, Yunnan Prov. (25.850, 98.861)	HQ235936	HQ235742	HQ235955	HQ235852	DQ126125*
	Schizothorax oconnori	Lasha, Xizang Prov. (29.665, 91.271)	HQ235937	HQ235743	HQ235959	HQ235854	AY463519*
	Schizothorax prenanti	Ya'an, Sichuan Prov. (30.021, 103.046)	HQ235938	DQ845910	HQ235951	HQ235849	AY954259*
	Schizothorax pseudaksaiensis	Yili, Xijiang Prov. (43.898, 81.303)	HQ235939	DQ845899	HQ235964	HQ235830	AF180827*
	Schizothorax waltoni	Chayu, Xizang Prov. (28.668, 97.473)	DQ366981	GQ406266	HQ235960	HQ235787	AY463518*
	Schizothorax wangchiachii	Panzhihua, Sichuan Prov. (26.588, 101.725)	HQ235940	HQ235744	HQ235943	HQ235813	AY954254*
	Schizothorax yunnanensis weiningensis	Luoping, Yunnan Prov. (24.891, 104.315)	HQ235941	HQ235745	–	HQ235851	HQ235775
	Schizothorax yunnanensis yunnanensis	Xiaguan, Yunnan Prov. (32.109, 118.773)	HQ235942	HQ235746	HQ235957	HQ235819	AY954252*

the ML tree was evaluated using the likelihood ratio test with likelihood values estimated with and without enforcing a strict molecular clock. In the absence of rate-constant model, we estimated the divergence dates using the penalized likelihood (PL) approach (Sanderson, 2002) as implemented in the program r8s version 1.7 (Sanderson, 2003), which relaxes the assumption of a strict molecular clock with a continuous autocorrelation of substitution rates across the phylogeny and allows tuning of rate variation via a cross-validation procedure.

Divergence date estimates were based on the tree topology obtained from the Bayesian analysis. In the PL method for estimating divergence times, a truncated Newton (TN) algorithm for optimizing the objective function and the additive penalty function were used. We used fossils to calibrate the ultrametric tree and to date internal nodes. The earliest fossil specimens of *Labeo*-like and *Barbus*-like fishes are known from Early Miocene, and thus a minimum age constaint (16.0 Mya) was assigned to the root of the subfamily Cyprininae (Stewart, 2001; Van Couvering, 1977). The fossil specimens of *Labeo* sp. in the Late Miocene were mapped as a minimum age constraint to the root of the labeonine clade (Stewart, 2009; Van Couvering, 1977), and the fossil specimens of *Barbus* sp. in the Late Miocene mapped as a minimum age constraint to the root of the barbine clade (Otero, 2001; Van Couvering, 1977). We fixed an age of 27.70 Mya to the origin of the subfamily Cyprininae because molecular clock studies suggested that the separation of the subfamilies Cyprininae and Leuciscinae occurred in the mid-Oligocene. Furthermore, the 95% confidence intervals for these estimated ages were also determined using the lengthy boostrap analysis. One hundred bootstrap pseudoreplicates were generated from the combined data matrix using SEQBOOT in Phylip 3.5c (Felsenstein, 1993). While keeping the tree fixed, for each pseudoreplicate the nodal depth (hence age estimates) of the nodes was estimated by ML with the preferred model of molecular evolution selected by ModelTest (Sanderson, 1997). For each node, the 95% confidence intervals of nodal ages were calculated on a distribution of 100 ages obtained by bootstrapping the concatenated data matrix.

Temporal variation in diversification rates

We employed temporal methods as implemented in the R program package LASER (Rabosky, 2006a) to address the specific hypotheses concerning diversification rates, such as when, how much, and where diversification rate shifts took place along the inferred Cyprininae phylogeny. The ingroup PL chronogram, the chronogram resulting from PL analysis of Bayesian tree with all non-Cyprininae taxa and duplicate species excised from the tree, was used to test for shifts in diversification rates.

The test was based on the birth-death likelihood analysis (BDL) (Rabosky, 2006b). The null hypothesis of constancy of diversification rates is tested using AIC. In the present study, a set of diversification models implemented in LASER, e.g. pure birth, birth-death, exponential density-dependent (DDX), logistic density-dependent (DDL), yule2rate, and yule3rate were considered for AIC tests. Only observed branching times were considered as possible shift points. The significance of the observed change in AIC (ΔAIC_{RC}) was assessed with the function yuleSim of LASER, which simulates 1,000 phylogenies with the same number of taxa under the null hypothesis of rate-constancy (pure birth) model.

Diversification rate shifts

We used the topological program SymmeTREE (Chan and Moore, 2005) to determine if diversification rate shifts exist across the Cyprininae. The topological methods compare the observed topological distribution of species' diversity to a distribution generated under the Yule model of an equal-rates Markov random-branching process. We performed six whole-tree tests of differential diversification rates: M_R, M_Π and M_Σ, $M_\Pi{}^*$ and $M_\Sigma{}^*$, and I_C with the program SymmeTREE, and these statistics are differentially sensitive to diversification rate variation at different node depths (Chan and Moore, 2002, 2005; Moore et al., 2004).

To detect and locate unusually rapid diversification rate shifts across the Cyprininae lineages, we used four "shift" statistics differing in power and bias, two likelihood ratio-based statistics (Δ_1 and Δ_2), the Slowinski and Guyer statistic (Chan and Moore, 2005) (SymmeTREE 1.1), and the relative cladogenesis statistic (Nee et al., 1992) (implemented in END-EPI 1.0 as the relative cladogenesis test).

Semilogarithmic plot of Lineage-through-time

We constructed a Lineage-through-time (LTT) plot of the ingroup PL chronogram without doublet species using the program GENIE 3.0. To evaluate the effects of incomplete taxon sampling on the slope of the empirical LTT plot, we generated 1,000 replicate phylogenetic trees with 1,132 extant taxa under a death-birth ratio of 0.5, and randomly pruned each tree to 123 taxa by using the program Phylogen 1.1. These 1,000 subsampled trees with 123 taxa were then used to construct a mean LTT curve and 95% confidence interval for comparison with the empirical LTT curve.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Tree resulting from Bayesian analyses of the concatenated gene sequences. Nodes for the key clades recognized are marked with abbreviated capitals referring to those in Table 1. Numbers near the nodes represent the values of posterior probability/bootstrap proportion/bootstrap proportion/decay indices.

Table S1 The six whole-tree tests of diversification rate variation in the cyprinid phylogeny by batch analysis of the bootstrap profile of trees from ML analysis under unpartitioned strategy using RAxML

Table S2 Results of fitting diversification models to the Cyprininae using Birth-Death Likelihood

Table S3 PCR primers (names, sequences and references) to amplify four mitochondrial and one nuclear DNA genes

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