

# Evolution of the Yangtze River reconstructed from the largest molecular phylogeny of Cyprinidae

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## Research Article

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# Abstract

The Yangtze River is the longest river in Asia, but its evolutionary history has long been debated, in particular the origin of the First Bend and formation of the Three Gorges. Diverse groups of endemic freshwater fishes have evolved in this river. Here we present the historical, spatiotemporal pattern of the endemic East Asian cyprinid clade based on the largest molecular phylogeny of Cyprinidae, including 1420 species and fossil records. Based on the evolution of egg types adapting to different hydrological conditions, we show that the ancestors of this endemic clade (laying adhesive eggs) were distributed in southern East Asia before ~24 Ma and subsequently dispersed to the Yangtze River basin to spawn semi-buoyant eggs at ~19 Ma. These results are consistent with the Yangtze River reversing its flow direction from southward to eastward to form the present river system around the Oligocene-Miocene boundary (~24–19 Ma). Some East Asian cyprinids evolved into fishes producing adhesive eggs again at ~13 Ma. This together with an increased net diversification rate, indicates that the river formed a potamo-lacustrine system during the Mid-Miocene. This new reconstruction of the history of the Yangtze River system through Cyprinidae phylogeny, together with the evolution of egg types in endemic East Asia cyprinids, improves the time resolution derived from geological studies.

## Significance Statement

The historical changes of the river system promote the diversification and dispersal of freshwater fish, whereas the phylogeographic dynamics of freshwater fish could be used to reflect the spatiotemporal evolution of rivers. By constructing the largest phylogenetic tree of Cyprinidae, reconstructing the ancestral state of egg type and historical distribution and estimating net diversification rate of endemic East Asian cyprinids, we showed the spatiotemporal patterns of the evolution of the Yangtze River system combined with fossil records and geological data. We biologically dated the age of the formation of the Yangtze River system around the Oligocene-Miocene boundary (~24–19 Ma), and indicated that the river probably had formed a huge potamo-lacustrine ecosystem by the Middle Miocene.

## Introduction

The Asian climate evolved from a planetary wind system in the Paleogene to a monsoon setting during the Neogene (1-3), linked in part to the uplift of the Qinghai-Tibetan Plateau. This same process led to the evolution of drainage systems in South and East Asia (4-6). The Yangtze River is the longest river in Asia, and its origin and evolution have attracted wide attention. It has been suggested that the upper reaches of the palaeo-Yangtze River (palaeo-Jinshajiang) originally flowed south from its source towards the South China Sea, but at some point diverted eastward in response to tectonic movements, and finally incised through the Three Gorges to form the modern Yangtze River (4,5,7,8). However, an exact understanding of the spatiotemporal evolution of the Yangtze River system has been challenging (8). Earlier geological surveys (*SI Appendix*, Tables S1 and S2) argued for the initial date when the palaeo-

Jinshajiang diverted eastward at the First Bend to range from the late Eocene (9) to the early Pleistocene (10). Likewise, ages for the incision of the Three Gorges range from the Eocene 40–45 Ma (11) to as recent as the Pleistocene, a few hundred thousand years ago (12), based on different sampling locations, dating methods and proxies. However, no robust biological evidence is available.

Compared to other river basins the Yangtze is one of the most diverse in terms of endemic species of freshwater fish worldwide (13,14). The historical changes in drainage basins governed the diversification and dispersion of freshwater fishes (15), and in turn, reconstruction of the phylogeographic dynamics of freshwater fishes could help constrain the spatiotemporal evolution of the river system (16,17). It is known that an endemic clade of East Asian Cyprinidae evolved while adapting to unique climatic and hydrological conditions under the influence of a strong East Asian monsoon during the uplift of the Qinghai-Tibetan Plateau (18). In this endemic clade, some fishes produce demersal eggs, while others produce adhesive or semi-buoyant eggs (Fig. 2a). Semi-buoyant eggs are considered to be a key trait used by East Asian cyprinids to adapt to monsoonal and large river environments (19,20). The development of semi-buoyant egg production, e.g., by the four major Chinese carp in the Yangtze River, required a long riverway (>500 km) with a fast flow (>0.5 ms<sup>-1</sup>) (21,22). The earliest fossils of *Ctenopharyngodon* and *Hypophthalmichthys* were found in the Lower Miocene of the Sihong Basin in the Yangtze River basin (23) (Fig. 2c). As these two carp spawn semi-buoyant eggs, the appearance of their ancestor can be used as a constraint on the formation of the modern Yangtze River. A few studies have focused on the evolution, radiation and key traits of endemic East Asian cyprinids that have adapted to the East Asian monsoon and large rivers (19,20), yet no attention has been given to the historical distribution and dispersal of cyprinids across East Asia, or the possible relationship between the spatiotemporal development of the Yangtze River and the evolution of egg types of endemic East Asian cyprinids.

We reconstructed the phylogeny of Cyprinidae based on the largest molecular data set currently available for cytochrome b genes from 1,420 Cyprinidae species belonging to nine subfamilies and 284 genera (*SI Appendix Data*). The topologies of phylogenetic trees obtained by Maximum likelihood and Bayesian inference analyses were almost consistent (Fig. 1), and these trees are also similar to those reconstructed by previous studies (24-27). By using fourteen calibration points including sixteen fossil records (*SI Appendix Text*), we estimated the divergence times for Cyprinidae (*SI Appendix Fig. S3*). Subsequently, we obtained the time-calibrated phylogeny of endemic East Asian cyprinids with different egg types (Fig. 2a) from the ancestral egg type reconstruction of Cyprinidae (*SI Appendix Fig. S5*). Based on the fossil records and the main distribution of the extant endemic East Asian cyprinids (*SI Appendix Data*), the ancestral distribution of this endemic clade was inferred (Fig. 2b-d). Combined with the evolution of different egg types and the historical distribution and dispersion of the endemic East Asian cyprinids, we estimated the age at which the southward-flowing palaeo-Jinshajiang first connected with the middle reaches via the First Bend and formed the Yangtze River system, including the potamo-lacustrine system in the middle and lower reaches of the Yangtze River.

# Results And Discussion

## The palaeo-Jinshajiang flowing southward in the Oligocene

Our results show that the ancestors of the endemic East Asian cyprinid clade, which produces adhesive eggs, appeared in the late Oligocene (~24 Ma; 95% credibility interval (CI): 22.3–26.7 Ma), including metzins, aphyocyprins and opsariichthyins (Fig. 2a). Combined with the fossils of *Ecocarpia ningmingensis* in the Ningming Basin, Guangxi Province (28), and the primary distribution of extant species of these cyprinids, we inferred that their ancestors were distributed in southern East Asia largely within the palaeo-Pearl River and palaeo-Red River before ~24 Ma (Fig. 2b). This implies that the modern Yangtze River had not yet been formed and that the palaeo-Jinshajiang likely flowed towards the south, connecting with a stream similar to the modern Red River.

The cyprinid fossils *Nanningocyprinus wui* and *Huashancyprinus robustispinus* found in Oligocene formations of the Nanning and Ningming Basins are also consistent with our results (29). Other biological and geological evidence suggests that the palaeo-Jinshajiang once flowed southward and probably connected through the palaeo-Red River into the South China Sea (*SI Appendix*, Table S1). The age of the river capture at Laojunshan near the first bend of the Yangtze River occurred prior to 24 Ma, maybe as early as the Early Oligocene as suggested by the change in sediment composition in the Red River delta, (30). Incision of the gorge in the First Bend area started between 20 and 30 Ma based on bedrock apatite (U-Th-Sm)/He thermochronology (31). Recently, studies using  $^{40}\text{Ar}/^{39}\text{Ar}$  mica dating and zircon U-Pb dating methods coupled with statistical analysis suggest that a major Paleogene river probably originated in the southeastern Qinghai-Tibetan Plateau and flowed through the Jianchuan Basin, extending to northern Vietnam during the late Eocene-Oligocene period, but disappearing by the early Miocene (32,33). Biological evidence from a time-calibrated phylogeny of only one fish genus (34–36) showed that the dating of the south-flowing palaeo-Jinshajiang is younger than that predicted in this study by using the endemic East Asian Cyprinidae.

Schizothoracine fishes commonly live on the Qinghai-Tibetan Plateau and surrounding area at an elevation of 1250–4750 m a.s.l. (37,38). In this study, the time-calibrated phylogeny of Cyprinidae (*SI Appendix*, Fig. S3) reveals that the schizothoracine fish endemic to the Qinghai-Tibetan Plateau did not appear before ~20 Ma (*SI Appendix*, Fig. S7). Combined with palaeontological evidence (39), we infer that the palaeo-elevation of the central Qinghai-Tibet Plateau was fairly low in the Oligocene, may not above 2000 m a.s.l. During the Eocene, reorganization of rivers did not occur because the southeastern part of the plateau was not uplifted significantly until the Oligocene (32). At the same time, southern East Asia was in a humid belt, while a broad arid belt stretched across central East Asia from west to east (1,40). These results indicate that the middle and lower reaches of the Yangtze River system had not yet been connected to the Jinshajiang. Our study provides new biological dating for the southern flow of the palaeo-Jinshajiang in the Oligocene.

## The formation of the Yangtze River close to the Oligocene-Miocene boundary

Fishes with semi-buoyant eggs consisting of squaliobarbins and hypophthalmichthyins existed in the Yangtze during early Miocene (~19 Ma; 95% CI: 17.1–21.3 Ma) (Fig. 2a). The earliest fossils of *Hypophthalmichthys*, *Ctenopharyngodon* and *Elopichthys* were found from the Lower Miocene of the Sihong Basin, Jiangsu Province (23). These results indicate that the endemic East Asian cyprinids dispersed to the position of the current Yangtze River and evolved into fishes laying semi-buoyant eggs by approximately 19 Ma (Fig. 2c), suggesting that the Yangtze River had reversed its flow direction eastward and formed the present drainage system before that time, close to the Oligocene-Miocene boundary (~24–19 Ma). The chemical weathering index mineral ratio chlorite/(chlorite + haematite + goethite) ( $C_{RAT}$ ) from Ocean Drilling Program (ODP) Site 1148 in the South China Sea (41) rapidly rose to a peak at approximately 19 Ma (Fig. 3b), indicating that the climate in East Asia became humid at that time, and abundant rainfall was conducive to the formation of a major Yangtze River system with high discharge.

Geological studies constrain the age of formation of the present Yangtze River system to 23–36.5 Ma based on  $^{40}\text{Ar}/^{39}\text{Ar}$  dating of basalts and U-Pb dating of zircon sand grains from the lower reaches of the Yangtze River and the appearance of evaporites and lacustrine sedimentation in the Jiangnan Basin (7). Detrital zircon U-Pb geochronology and heavy mineral analysis from the Cenozoic sediments of the Jiangnan Basin define their provenance and indicate that the age of incision of the Three Gorges must have postdated 32 Ma. The best date for initiation of the modern river is likely after the ~24.6 Ma unconformity (42). These results are close to the date of Yangtze River formation estimated from the timing of divergence of the semi-buoyant egg group in our study. Due to using different sampling locations, dating methods and proxies, the ages of the connection and formation of the Yangtze River system were incompatible with other geologically based models (*SI Appendix*, Table S2).

The formation of the current Yangtze River system hindered gene flow of some terrestrial species between the north and south sides of the mainstream, resulting in genetic diversification and speciation. The divergence dating of the primitively segmented spider genera *Sinothela* and *Ganthela*, which are distributed on the north and south sides of the Yangtze River, was estimated to be 13–30 Ma, which is consistent with the suggested formation the modern Yangtze River system before the Miocene (43). This divergence timing has a much broader range than we inferred, probably due to fewer species and the lack of fossil calibrations.

In addition, the specialized schizothoracine fishes mostly live in the Qinghai-Tibetan Plateau at an elevation of >2750 m a.s.l (37,38). Based on the results of the time-calibrated phylogeny of schizothoracine fishes (*SI Appendix*, Fig. S7), the timing of divergence between primitive and specialized grades was likely at ~18 Ma, indicating that the Qinghai-Tibetan Plateau had reached a high elevation in the early Miocene. The surface of the southeastern Qinghai-Tibetan plateau was uplifted when ductile lower crust beneath the central plateau flowed towards the plateau margin from the late Oligocene to the early Miocene (44,45). At the same time large-scale strike-slip faults linked to extrusion of crustal blocks from Tibet by the colliding Indian block resulted in the reversal or capture of river systems (4,7).

Therefore, the Yangtze River diverted its flow from being towards the south to eastward and incised through the Three Gorges to form the modern river system at that time.

### **Formation of the potamo-lacustrine ecosystem in the Yangtze River in the middle Miocene**

Formation of a potamo-lacustrine complex ecosystem greatly promoted fish diversification (46). Previous studies have mainly focused on the formation mechanisms and ages of several of the modern lakes in the Yangtze River basin (47,48), while the earliest formation of the potamo-lacustrine ecosystem remains unclear.

Based on the result of the rate-through-time plot from the Bayesian Analysis of Macroevolutionary Mixtures program (Fig. 3a), the net diversification rate of the endemic East Asian cyprinids increased quickly at approximately 13 Ma. This result implies that the drainage network was rich in the Yangtze River basin, and provided a large number of niches, facilitating rapid radiation and dispersal of fishes. In the middle Miocene (~13 Ma; 95% CI: 11.4–14.6 Ma), fish laying adhesive eggs arose again, including xenocyprins and cultrins (Fig. 2a). This finding indicates that to adapt to the lake environment, endemic East Asian cyprinids evolved into fishes spawning adhesive eggs that attached to aquatic plants to develop. These results suggest that the potamo-lacustrine ecosystem of the Yangtze River had appeared by that time (Fig. 2d). Coincidentally, the chemical weathering index  $C_{\text{RAT}}$  of sediment at ODP Site 1148 in the South China Sea, which is related to humidity and temperature peaked at ~13 Ma (Fig. 3b) (41), which indicates that the climate in East Asia was humid at that time. Strong precipitation would have sustained a potamo-lacustrine ecosystem in the Yangtze River, greatly increasing species diversification.

In summary, we used the spatiotemporal evolutionary pattern of endemic East Asian cyprinids from the largest molecular phylogenetic tree of Cyprinidae, fossil records and information on egg type evolution while adapting to varied hydrologic conditions to reconstruct the formation history of the Yangtze River system. Our results indicate that the ancestors of East Asian cyprinids were confined to the south of East Asia between the palaeo-Pearl and palaeo-Red rivers during the Oligocene, prior to formation of the Yangtze River system. At that time the palaeo-Jinshajiang flowed southward to the South China Sea roughly along the course of the modern Red River. Endemic East Asian cyprinids had dispersed to the Yangtze River basin and evolved into fishes laying semi-buoyant eggs by ~19 Ma, which suggests that the Yangtze River system had formed by that time in response to regional surface uplift, large strike-slip tectonism and climate change. The formation of the Yangtze River is constrained to be around the Oligocene-Miocene boundary (~24–19 Ma). Notably, the endemic East Asian cyprinids evolved into fishes spawning adhesive eggs again by approximately 13 Ma, coinciding with a rapid increase in the net diversification rate of this endemic clade and a peak in the intensity of the East Asian summer monsoon (41), indicating that the Yangtze River system probably had developed into a potamo-lacustrine ecosystem by the middle Miocene. Our studies constrain the ages of important geological events during the evolution of the Yangtze River from a biological perspective, helping us to understand the evolutionary history of the Yangtze River system.

# Materials And Methods

**Data collection and processing.** Information on the scientific names, taxonomic position, distribution and egg type of Cyprinidae was obtained from FishBase ([www.fishbase.org](http://www.fishbase.org)), Catalog of Fishes (<http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp>) and additional dedicated publications (*SI Appendix Data*). A total of 1423 cytochrome *b* sequences from 284 genera of Cyprinidae and three outgroup taxa were collected by GenBank (accession numbers of all the sequences are listed in *SI Appendix Data*). While sequences were aligned using MAFFT version 7, ambiguous regions in alignments were removed using Gblocks v.0.91 (49).

**Phylogenetic analyses.** The phylogenetic analyses were conducted with Maximum likelihood (ML) and Bayesian inference (BI) in RAxML v. 8.2.12 (50) and MrBayes v.3.2 (51), respectively. The ML analyses were implemented under a GTRGAMMA model with 100 rapid bootstrap inferences using a thorough ML search. For BI analyses, the best-fitting nucleotide substitution model GTR+F+I+G4 was calculated with ModelFinder in PhyloSuite (52). Two independent runs were performed through 20,000,000 generations with four Markov chains. The first 25% of trees were removed as burn-in. Chain convergence was inspected in Tracer 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>), and the results with an effective sample size (ESS) for each parameter >200 were accepted. A consensus tree was produced.

**Divergence time estimation.** Based on the optimal ML tree topology obtained with RAxML v 8.2.12 (50), penalized likelihood dating analysis was conducted in treePL (53) to estimate divergence time. We sorted the available fossils from the literature and selected sixteen fossils as fourteen calibration points (*SI Appendix Text*). To identify the appropriate level of rate heterogeneity in the phylogram, cross-validation analysis was conducted in treePL while testing 37 smoothing parameter values from 1018 to 10–18. To calculate the confidence intervals for the dating estimates of each node, 100 bootstrap replicates were generated by RAxML, with topology fixed to the best ML phylogram but with varying branch lengths. We then conducted treePL on these 100 replicates. Age statistics of all nodes were summarized with TreeAnnotator v.1.10.4 (54).

**Rate-through-time analysis.** The net diversification rate of cyprinids was inferred based on the chronogram of Cyprinidae using the program Bayesian Analysis of Macroevolutionary Mixtures (BAMM) v2.5 (55). The BAMM analysis was run for 100 million generations at a Poisson rate prior to 0.1, sampling event data every 10,000 generations. Prior distributions were set based on the *setBAMMPriors* function in the BAMMtools R package (56). The first 20% of samples were discarded as burn-in. The rate-through-time plot of the endemic East Asian cyprinids was extracted and visualized by using BAMMtools from the Cyprinidae data set.

**Ancestral state reconstruction.** The egg types of cyprinids from 507 species were coded as A (adhesive), B (bivalve), C (demersal), D (nesting), and E (semi-buoyant) (*SI Appendix Data*). The ancestral state of egg types was reconstructed using BI. BI analysis was implemented in RASP v.4.0 (57) with Bayesian Binary Markov Chain Monte Carlo (BBM). Ten MCMC chains were run simultaneously for 5,000,000 generations with the JC (Jukes-Cantor) fixed model, and the maximum number of areas was set to 1.

# Declarations

## Data availability

We have chosen not to deposit the data at this time but declare that data supporting the findings of this study are available within this article and its Supporting Information (*SI Appendix*), and all additional data are available from the corresponding author on request.

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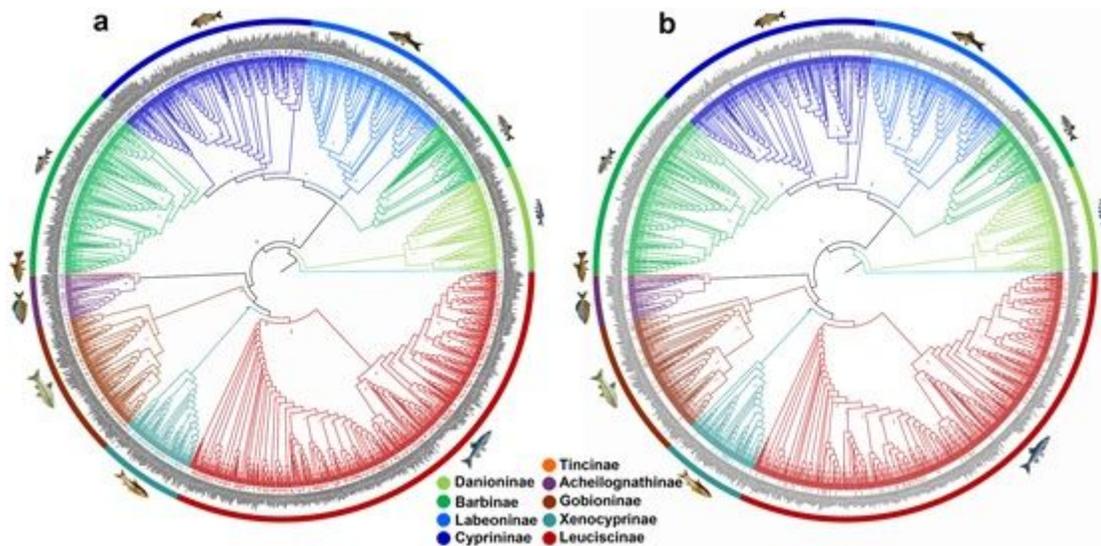
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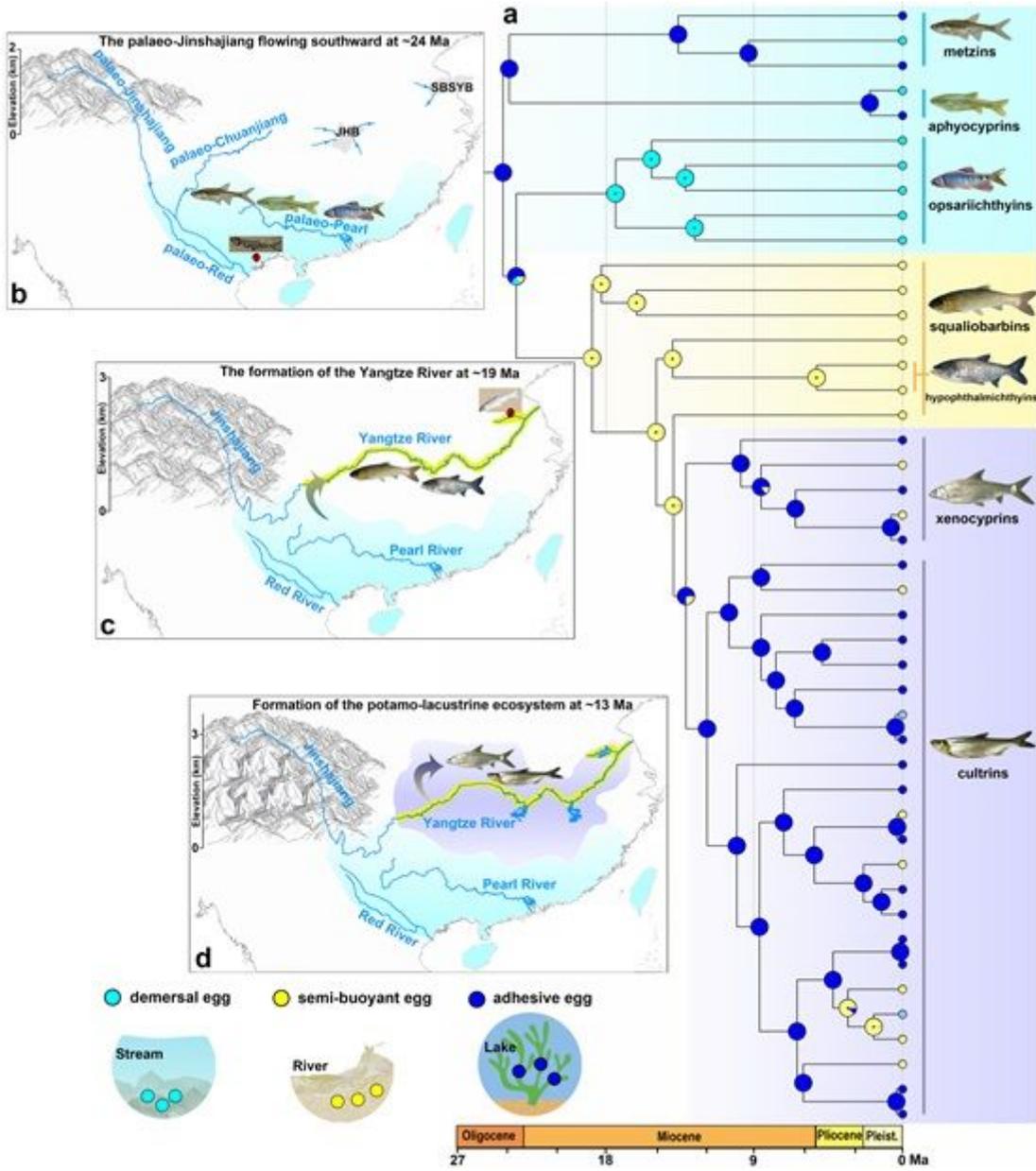
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## Figures



**Figure 1**

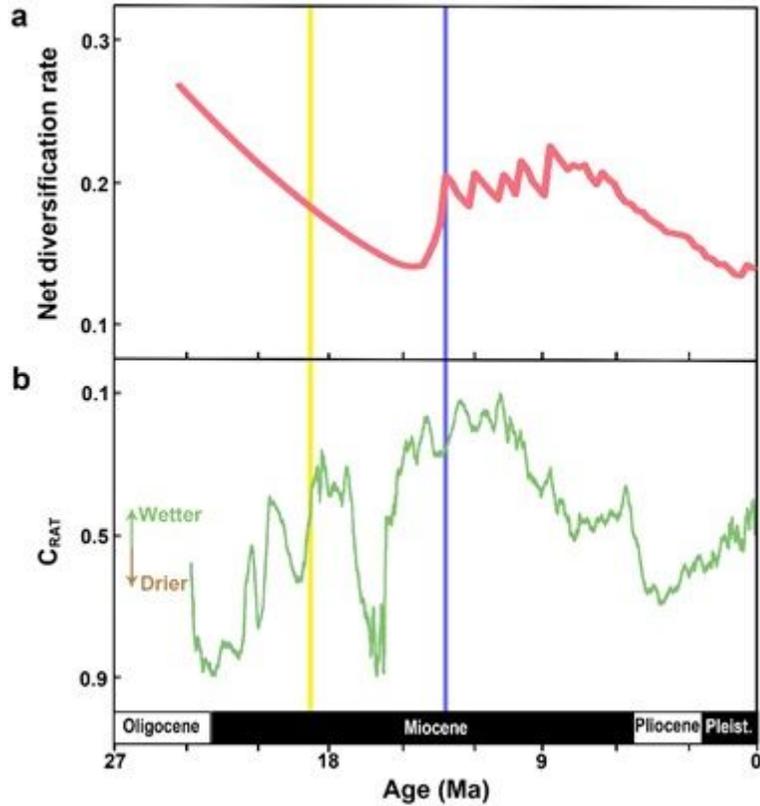
Phylogeny of 1420 Cyprinidae species and three Cobitidae species inferred from cytochrome b genes based on Maximum likelihood and Bayesian inference analyses (see details in SI Appendix, Figs. S1 and S2). a, The topology of the phylogenetic tree obtained by maximum likelihood analysis. The numbers beside the nodes are the bootstrap proportions. b, The topology of the phylogenetic tree obtained by Bayesian inference analysis. The numbers beside the nodes are Bayesian posterior probabilities.



**Figure 2**

Egg type evolution and schematic diagram of the historical distribution and dispersal of the endemic East Asian clade of Cyprinidae under the development of the Yangtze River system in response to uplift of the Qinghai-Tibetan Plateau. a, Egg type evolution of the endemic East Asian cyprinids (see details in SI Appendix, Fig. S6). b-d, Schematic diagram of the historical distribution and dispersal of endemic East Asian cyprinids during the development of the Yangtze River system during uplift of the Qinghai-Tibetan Plateau. Different coloured regions represent different groups (a) and their ancestral distribution (b-d). Red dots indicate fossil sites of endemic East Asian cyprinid fish, including the Ningming Basin, Guangxi Province (b, 1) and Sihong Basin, Jiangsu Province (c, 2). JHB, Jiangan Basin; SBSYB, Subei-South Yellow Sea Basin (b). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the

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**Figure 3**

Net diversification rate and chemical weathering index CRAT as a function of time. a, The rate-through-time plot of the endemic East Asian cyprinids (see details in SI Appendix, Fig. S4). b, The chemical weathering index CRAT of ODP Site 1148 in the South China Sea as a function of time (modified from Clift et al. (41)). The yellow line indicates the appearance of the semi-buoyant egg group at ~19 Ma. The purple line represents that the fishes with adhesive eggs appeared again, and the net diversification rate of the endemic East Asian cyprinids increased rapidly at ~13 Ma.

## Supplementary Files

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