# Phylogeny, evolution, and biogeography of Asiatic Salamanders (Hynobiidae)

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**We sequenced 15 complete mitochondrial genomes and performed comprehensive molecular phylogenetic analyses to study the origin and phylogeny of the Hynobiidae, an ancient lineage of living salamanders. Our phylogenetic analyses show that the Hynobiidae is a clade with well resolved relationships, and our results contrast with a morphology-based phylogenetic hypothesis. These salamanders have low vagility and are limited in their distribution primarily by deserts, mountains, and oceans. Our analysis suggests that the relationships among living hynobiids have been shaped primarily by geography. We show that four-toed species assigned to *Batrachuperus* do not form a monophyletic group, and those that occur in Afghanistan and Iran are transferred to the resurrected *Paradactylodon*. Convergent morphological characters in different hynobiid lineages are likely produced by similar environmental selective pressures. Clock-independent molecular dating suggests that hynobiids originated in the Middle Cretaceous [≈110 million years ago (Mya)]. We propose an ''out of North China'' hypothesis for hynobiid origins and hypothesize an ancestral stream-adapted form. Given the particular distributional patterns and our molecular dating estimates, we hypothesize that: (*i*) the interior desertification from Mongolia to Western Asia began ≈50 Mya; (*ii*) the Tibetan plateau (at least on the eastern fringe) experienced rapid uplift ≈40 Mya and reached an altitude of at least 2,500 m; and (*iii*) the Ailao–Red River shear zone underwent the most intense orogenic movement ≈24 Mya.**

mitochondrial DNA | phylogenetics | homoplasy | Tibetan Plateau

The Asiatic Salamanders, Hynobiidae, represent an early branch of the caudate lineage (1). All living species (≈50, in seven to nine genera; http://amphibiaweb.org) occur in Asia. Hynobiids are closely related to the family Cryptobranchidae, with which they form the suborder Cryptobranchoidea. In comparison with other living salamanders, hynobiids are thought to resemble the most recent common ancestor of all salamanders because of three traits that are regarded as ancestral: external fertilization, an angular bone in the lower jaw, and large numbers of microchromosomes (1–3). Although usually thought to be monophyletic, the Hynobiidae also has been considered a paraphyletic stem group (4). Fossil hynobiids are known from the Late Miocene [≈5 million years ago (Mya)] (5), whereas their relatives, cryptobranchids, can be traced back to the Jurassic (≈160 Mya) (6). Remarkably, recent fossil findings of salamanders from the Early Cretaceous of North China show strong similarities with the Hynobiidae with respect to many skeletal features (7–9). This finding raises the questions of where and when hynobiids originated and what relationship there is between them and their fossil relatives. To answer these questions, a robust phylogenetic hypothesis of living hynobiids is required. Apart from a tentative phylogeny based on 23 morphological characters (Fig. 1; ref. 10), and another based on an unpublished data set (11), no well supported phylogenetic hypothesis exists.

Although hynobiids are found throughout the Asian continent, their distribution is discontinuous. Given their inability to readily cross deserts and mountains, orogenic movements, as well as inland desertification, may well play important roles in shaping the distribution of lineages. After the collision of India with Asia in the early Cenozoic, the Tibetan plateau began to uplift (12–15). This uplift greatly modified environments all over the Asian continent and has been invoked as the main driving force behind long-term Cenozoic climate change (16, 17). Hynobiids likely experienced effects from this great geologic event because their current distributions are related to geological features such as the Tibetan plateau and its concomitants: loess and deserts. In turn, an accurate estimate of phylogeny and divergence times of living hynobiids will provide clues concerning the effects of geologic events.

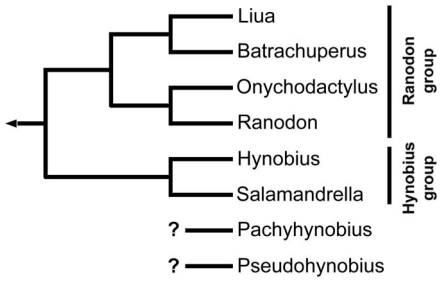


**Fig. 1.** Traditional relationships among the genera of the family Hynobiidae based on 23 morphological characters (10). The living hynobiids were divided into two groups (*Hynobius* and *Ranodon*). The genera *Pachyhynobius* and *Pseudohynobius* were not included in that study, and their phylogenetic position relative to the other genera is uncertain.

In this study, we sequenced 15 previously unreported complete mitochondrial genomes of hynobiids. By combining these sequences with published amphibian mitochondrial genomes, we present a comprehensive molecular phylogeny for living hynobiids. Moreover, by using molecular clock-independent approaches for inferring dating information from molecular phylogenies (18), a timescale for events in hynobiid evolution is given.

## Results

The final DNA alignment contains 14,311 nucleotide sites for the 18 taxa listed in Table 1, which is published as supporting information on the PNAS web site. Of these sites, 7,494 are constant, 4,712 are informative for parsimony, and 2,105 are otherwise variable. Maximum parsimony (MP), neighbor-joining (NJ), Bayesian inference, and maximum likelihood (ML) recover nearly identical topologies but vary in the level of support for some nodes (Fig. 2). The monophyly of all hynobiid salamanders with respect to

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Abbreviations: Myr, million years; Mya, million years ago; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor-joining.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ333808–DQ333822).

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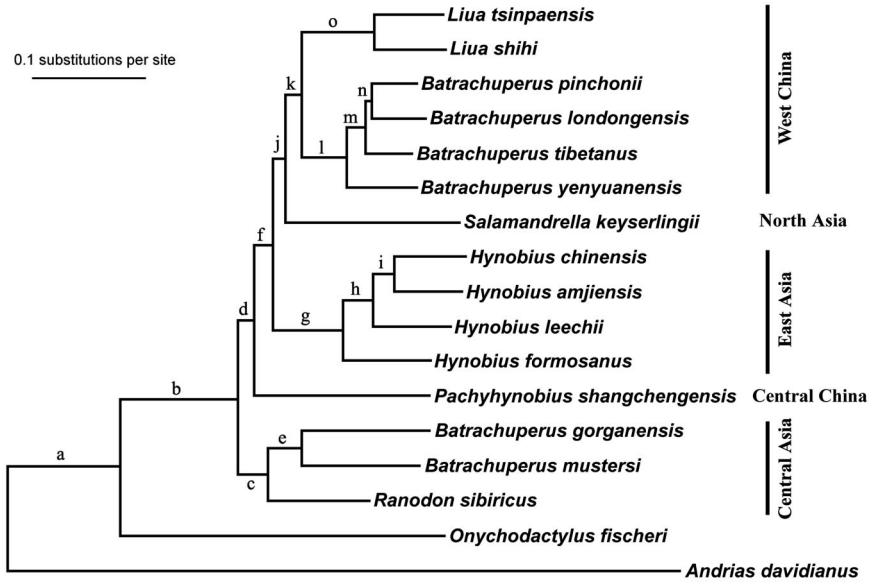


**Fig. 2.** Phylogenetic relationships of living hynobiid salamanders inferred from analyses of mitochondrial genome sequences. Branches with letters have branch support values given below the tree for MP, NJ, unpartitioned Bayesian (UBA), and partitioned Bayesian (PBA) methods. Outgroup species are not shown. Branch lengths were estimated by using ML inference. (Scale bar represents 0.1 substitutions per site.) –, branch was unresolved (i.e., values < 50).

cryptobranchid salamander is supported by all analyses (Fig. 2, node a). At the base of the tree, *Onychodactylus* is the sister group of all other living hynobiids (Fig. 2, node b). The traditional grouping of hynobiids according to morphological characters (Fig. 1) is not supported. *Batrachuperus* is diphyletic, with species sorting into two clades, a Central–Western Asia group (Fig. 2, node e) and a West China group (Fig. 2, node l), despite the morphological support for this genus (19). The central Asian species cluster with *Ranodon sibiricus* (Fig. 2, node c), which has a central Asian distribution but is morphologically distinct. All hynobiid salamanders distributed in West China form a clade (Fig. 2, node k), despite substantial differences in morphology and life history. Surprisingly, *Salamandrella* clusters with mountain-type hynobiids (*Batrachuperus* and *Liua*) of West China (Fig. 2, node j), although it is primarily a species of the flat lowlands. The sister taxon of all of the West China hynobiids, the East Asian *Hynobius*, and the North Asian *Salamandrella*, is *Pachyhynobius shangchengensis* from Central China, but support levels are lower than for other major nodes (Fig. 2, node d).

The log-likelihoods (lnL) of the phylogenetic tree were lnL = −102791.83925 without a molecular clock constraint and lnL = −102908.32235 under the global clock, rooted with *Andrias* and *Paramesotriton*. A likelihood ratio test significantly rejected a global molecular clock for this data set ($P < 0.001$). To account for evolutionary rate variation among hynobiid lineages, we estimated relative nodal ages with a relaxed clock by using the penalized-likelihood and Langley–Fitch (local molecular clock) algorithms implemented in R8S (18). Both algorithms for estimating divergence times gave similar results; we report only those derived by the penalized-likelihood methods (Fig. 3). Our results show that the crown group of living hynobiids originated in the Middle Cretaceous (≈110 Mya). The mass generic diversification of living hynobiids occurred after the end-Cretaceous Mass Extinction event (≈65 Mya). The diversification of hynobiids distributed in West China began ≈40 Mya. Divergence of terminal taxa was initiated 25–15 Mya, within the early Miocene.

## Discussion

**Trait Evolution in Hynobiids: Extensive Homoplasy.** Hynobiids have either stream-type (*Onychodactylus*, *Ranodon*, *Paradactylodon*, *Batrachuperus*, *Pseudohynobius*, *Liua*, and some *Hynobius* species; we infer that the poorly known *Pachyhynobius* is here as well) or pond-type (*Salamandrella* and most *Hynobius* species) life histories. Stream-type species live in streams or close to streams, and their larvae develop in running water; pond-type species live in humid lowlands, and their larvae develop in still water. The traditional division of hynobiids into *Hynobius* and *Ranodon* groups based on features of dentition, length of larval state, numbers of eggs laid, and fontanelle (ref. 10; see Fig. 4 for details) is not supported from our ancestral state reconstruction analysis, which requires that characters in *Salamandrella* and some species of *Hynobius* evolved independently.

Identical characters in different hynobiid lineages may represent adaptive convergence. Stream-type hynobiids usually feed underwater and prey mainly on aquatic arthropods, which they capture by a sudden bite, or by suction feeding in the water (20) (Fig. 4). Transversely oriented vomerine teeth may hinder escape of prey when water is released from the mouth. Pond-type species (*Salamandrella* and most species of *Hynobius*) mainly feed on small terrestrial invertebrates, which they capture with tongue movements that deliver the prey deep within the mouth, where they are held by the posteriorly directed vomerine teeth (20). Similar types of vomerine teeth are present in plethodontid and salamandrid salamanders that use the tongue for prey capture.

Differences in larval duration and numbers of eggs laid also may be attributable to adaptive convergence (Fig. 4). The breeding habitats of pond-type species are subject to many risks for larvae, including early drying, environmental hypoxia, predation, food deficiency, and winter freezing, which favor early metamorphosis. Species with stream-type adaptations are mainly aquatic and lay relatively few but larger eggs in streams, which typically are well oxygenated and permanent, and rocky substrates provide refuge from predators while serving as good habitats for prey. Larvae of stream adapted species are typically perennial (10).

The arrangement of anterior cranial bones has been used in earlier studies of hynobiid evolution, in particular the relationship of the nasal bones to each other and to surrounding bones, and the

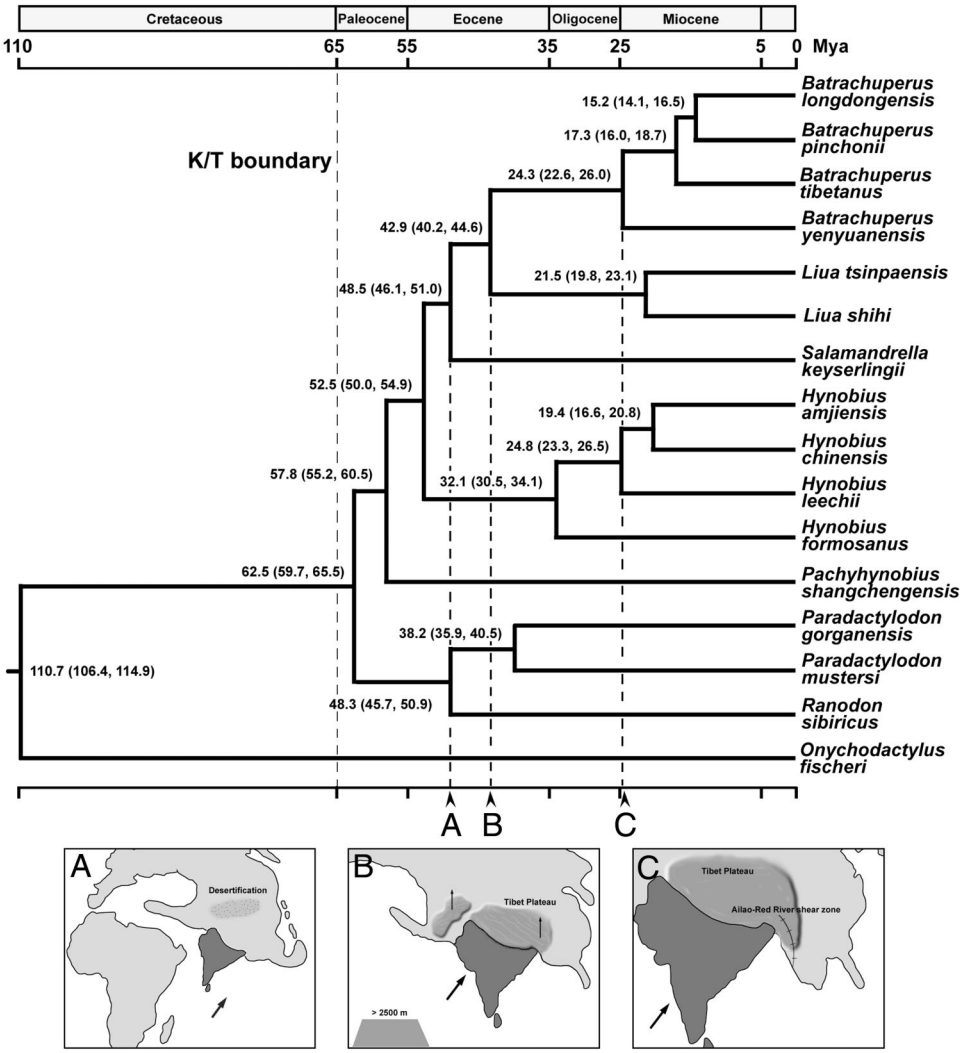


**Fig. 3.** Molecular tree topology of hynobiids combined with dating of the phylogenetic nodes. (*Upper*) Branch lengths are proportional to divergence times. Numbers above the nodes are the mean estimated divergence time (in Mya). Numbers in parentheses represent 95% credibility intervals. The hynobiids originated in the Mid-Cretaceous but began their major diversification after K/T boundary. Three important time points during the evolution of hynobiid are indicated by black triangles. The three geologic events corresponding to these time points are illustrated below the tree. (*A*) Interior desertification of Asia took place by ≈50 Mya. (*B*) Tibetan plateau (at least on the east fringe) experienced rapid uplift process ≈40 Mya and reached at least an altitude of 2,500 m. (*C*) The most intense orogenic movement of the Ailao–Red River shear zone took place 24 Mya.

fontanelle that appears between them and behind the premaxillary bones (10). When nasal bones are in close contact, no fontanelle is present (condition in *Pachyhynobius*, *Hynobius*, and *Salamandrella*, all deeply nested in our phylogeny). This condition has been considered ancestral for hynobiids (10). However, our results suggest that the ancestral state for hynobiids is presence of the fontanelle ($P = 0.87$; Fig. 4). Such a fontanelle is present in the Cretaceous fossil salamanders *Liaoxitriton* (8) and *Jeholotriton* (9), which may be close relatives of living hynobiids. Loss of the fontanelle may reflect increased ossification in more deeply nested lineages.

Noble (21) believed that the common ancestor of hynobiids was a pond-type species like *Hynobius*. Our results suggest that stream adaptation is likely to be ancestral in the Hynobiidae ($P = 0.94$; Fig. 4). *Salamandrella* and some *Hynobius* may have evolved several traits independently when their ancestors invaded pond habitats (Fig. 4).

Hynobiids have large numbers of chromosomes, including many microchromosomes ($2n = 40–78$). Cryptobranchids are similar in these respects ($2n = 60$). The basal split (Fig. 2, node a) separates *Onychodactylus* ($2n = 78$) from the remaining species, whose ancestor we infer to have had relatively high numbers ($2n = 66–68$). Microchromosomes have been lost in noncryptobranchoid salamanders, and chromosome numbers have stabilized at relatively low numbers ($2n = 24–28$) of relatively large, biarmed structures in other salamanders. Parallel evolution within the Hynobiidae is seen in different deeply nested hynobiid taxa, for example in *Hynobius* ($2n = 40–58$) and in *Pseudohynobius flavomaculatus* ($2n = 52$) (see *Appendix*, which is published as supporting information on the PNAS web site).

**Hynobiid Origins and Diversification.** The earliest fossil record of hynobiids is in the late Miocene of Europe (≈5 Mya) (5). Our molecular dating results indicate that hynobiids began diversifying much earlier, however, in the Middle Cretaceous (≈110 Mya; Fig. 3), a time when the present-day North American continent was still partly connected to the European continent. However, no fossil or extant hynobiids are known from North America, which makes a European origin for hynobiids unlikely. Recently, many fossils related to hynobiids have been reported from North China (see Fig. 5 for fossil localities): *Chunerpeton* (ref. 6; Cryptobranchidae, Middle Jurassic; ≈161 Mya), *Liaoxitriton* (refs. 7 and 8; close to living hynobiids in skeletal anatomy; Early Cretaceous), and *Jeholotriton* (ref. 9; possibly a cryptobranchoid; Early Cretaceous). Moreover, the basal-most divergence within the hynobiids separates *Onychodactylus*, which is also distributed in northern China, from other genera. Hence, under a parsimonious algorithm, we hypothesize an origin of hynobiids in what is present-day northern China (Fig. 5).

After the appearance of the common ancestors of living hynobiids in the Middle Cretaceous (≈110 Mya), early hynobiids began their dispersal into the present-day Eurasian continent from North

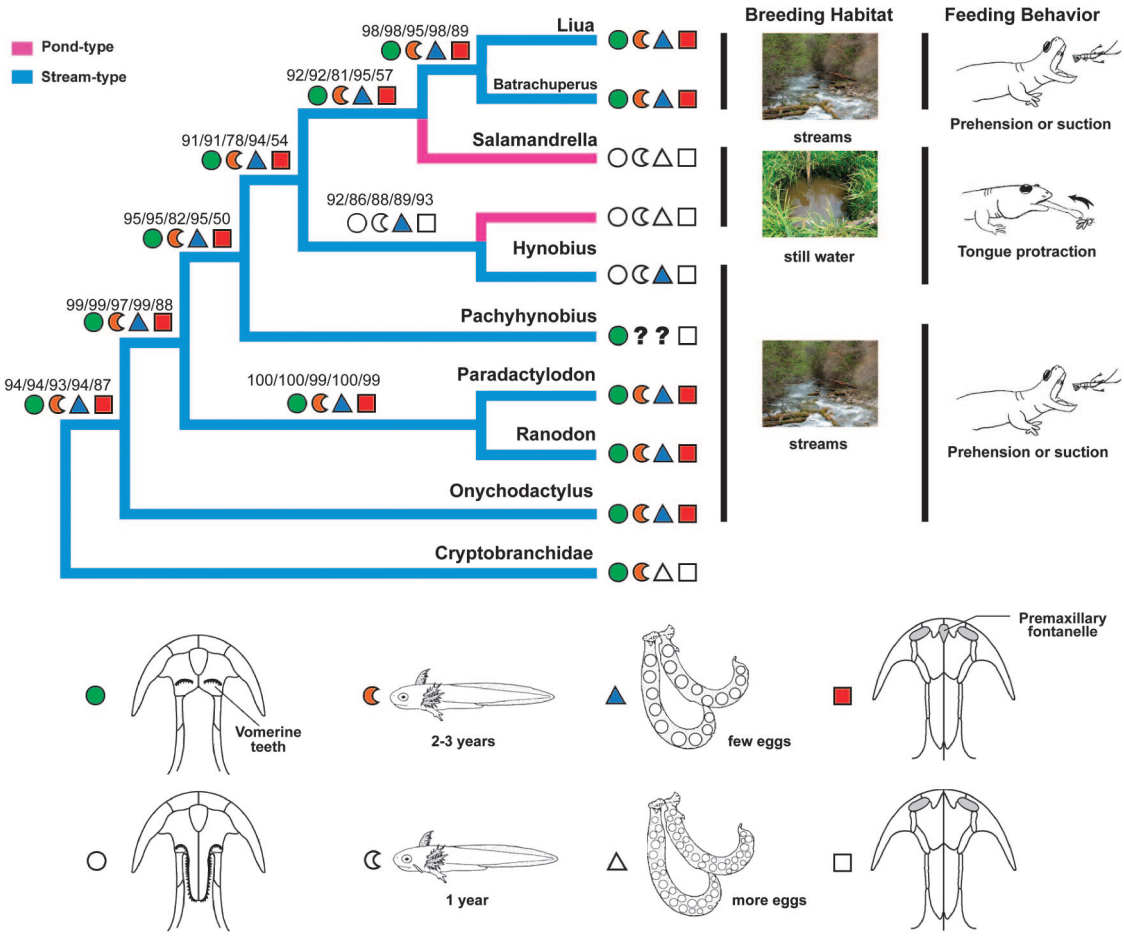


**Fig. 4.** Ancestral state reconstruction for five important traits in hynobiids under the present molecular phylogeny (Cryptobranchidae as outgroup). The five traits are life history (stream-type, blue line; pond-type, pink line), dentition (green circle, transversely oriented; open circle, posteriorly directed), length of larval state (orange moon, long larval state; open moon, short larval state), numbers of eggs laid (blue triangle, less eggs; open triangle, more eggs), and premaxillary fontanelle (red square, present; open square, absent). Numbers along the internodes are posterior probabilities for the five traits under the same order mentioned above. Different living habitats seem to impact the selection of feeding and breeding behaviors among hynobiid adults, causing adaptive convergence to some degree. Ancestral state reconstruction was performed by using MRBAYES (Version 3.1) (1,000,000 generations).

China. During this period the continent was relatively flat and humid. Perhaps these dispersing species were semiaquatic in habits and were able to disperse deep into the westernmost regions, as evidenced by fossil records of hynobiids from the Miocene of Europe (5). Further dispersal into North America was inhibited because the breakup of Laurasia had been well established (≈50 Mya) when hynobiids reached the European region. The most important diversification of hynobiids began after the Cretaceous–Tertiary boundary, indicating an extensive change both in climate and physiognomy. Later inland desertification events in Mongolia and Central Asia cut off genetic exchange between some hynobiid groups, resulting in the *Ranodon-Paradactylodon* split and the separation between *Salamandrella* and other hynobiids in West China. The first uplift of the Tibetan plateau (≈40 Mya), accompanied by the rise of many high mountains, greatly modified the physiognomy of West China (12). These mountains became geographic barriers to hynobiids, resulting in differentiation. West China thus became an important diversification center for stream-type hynobiids (Fig. 5), associated with mountainous regions.

**Biogeographic Implications of the Geological Evolution of Asia.** Hynobiids are dependent on humid habitat and have low vagility (19). Accordingly, high mountains and deserts are barriers to their dispersal. The current discontinuous distribution of living hynobiids across the Asian continent, with a heavy concentration in eastern Asia, is the result of limited dispersal opportunities throughout Tertiary and Quaternary times.

Onset of Asian desertification began at least 22 Mya (22). The distribution of hynobiids may reflect this event, which probably separated *Salamandrella* from its sister clade, the stream-adapted genera (*Batrachuperus* and *Liua*) of western China and separated *Ranodon* from *Paradactylodon* in central Asia (Fig. 5). The split between *Ranodon* and *Paradactylodon* and that between *Salamandrella* and the group of *Batrachuperus* and *Liua* took place nearly in the same period (≈46–51 Mya) (Fig. 3, node A), a time a little later than the initiation of the India–Asia collision (≈55 Mya) (15). Geologically, the India–Asia collision altered land–sea distribution of the Asian continent and reduced precipitation coming from the Indian Ocean. Our dating results suggest that the India–Asia collision may be a driving force that triggered inland desertification from Mongolia to Central and Western Asia 50 Mya (Fig. 3*A*).

The split between *Batrachuperus* and other mountain hynobiids (*Liua* and *Pseudohynobius*) may have been the outcome of active orogenic movements on the eastern edge of the Tibetan plateau. The estimated time for this event (≈40–45 Mya) is consistent with the previous geological hypothesis that the first rapid uplift of the Tibetan plateau took place along its eastern border ≈40 Mya (12). The diversification of central Asian species of *Paradactylodon* began ≈36–41 Mya, suggesting intense orogeny in central Asia at that time (Fig. 3 *Upper*, node B). In the mountains of western China,


**Fig. 5.** Current distribution of living hynobiids across the Asian continent. The distribution area of living hynobiids is indicated in green. The locality for related fossil taxa is indicated as a black triangle. North China is hypothesized to be the center for the origin of hynobiids. West China is considered as a secondary diversification center (mainly for mountain-type hynobiids). The Tibetan plateau and its concomitants, loess and deserts, have played important roles in shaping distribution patterns.

*Batrachuperus* is distributed to the west, along the eastern edge of the Tibetan plateau, whereas *Liua* and *Pseudohynobius* are distributed more to the east, in mountainous areas outside the Tibetan plateau. In addition, the critical altitude for these mountain hynobiids is ≈2,500 m; only *Batrachuperus* can live above this altitude. Accordingly, we hypothesize that *Batrachuperus* separated from the other mountain hynobiids to the east as the eastern fringe of the Tibetan plateau reached about the 2,500 m level, after the first uplift ≈40 Mya (Fig. 3*B*). This inference is compatible with the recent geologic result that the surface of Tibet has been at an elevation of >4 km for at least the past 35 million years (Myr) (23).

The genus *Batrachuperus* is of Tibetan origin because these salamanders occur only on the eastern border of the Tibetan plateau. According to our timescale, the diversification of *Batrachuperus* took place from 25 to 15 Mya (Fig. 3), which implies that species formation is associated with active orogeny in Tibet during that time. This result is compatible with the geological hypothesis that rapid uplift took place in Tibet by 20 Mya (24, 25). The Ailao–Red River shear zone, from southeastern Tibet through Yunnan to the South China Sea, was an extrusion boundary of the Indochina block during the Indo-Asian collision (Fig. 3*C*). This zone was active between 35 and 17 Mya; the main geologic age for this zone was ≈23 Myr (26). Among all *Batrachuperus* species, the southernmost *B. yenyuanensis* is the only species distributed in this zone, and it can dwell at altitudes up to 4,000 m. The estimated divergence time of *B. yenyuanensis* from other *Batrachuperus* species is ≈24 Myr (Fig. 3 *Upper*, node C); this result supports the geological hypothesis and further suggests intense orogeny of this region 24 Mya.

**Taxonomic Implications.** Although some authors have questioned the monophyly of the Hynobiidae (4), our results strongly support its status as a clade and as a taxon. However, although the species assigned to *Batrachuperus* from Central Asia share three significant traits with Chinese members of the genus [aquatic habits, large premaxillary fontanelle, and four toes on the hind limb (19, 27)], *Batrachuperus* is diphyletic, and the Central and Western Asian species are relatives of *Ranodon.* However, these species are biologically distinct from *Ranodon*, which is only semiaquatic and has five toes in the hind limb, and they are not appropriately assigned to that genus. The species *B. gorganensis* was once assigned to a monophyletic *Paradactylodon* (28), but this genus has not been recognized (27). We here resurrect the genus *Paradactylodon* and assign to it three species [*mustersi*, *persicus*, and *gorganensis*; the last may be a synonym of *persicus* (29)].

**Phylogenetic History of the Hynobiid Lineage.** A Mesozoic origin of salamanders has long been proposed, based on the distribution of fossils and earth history (6, 30) and molecular data (31, 32); our results are consistent with these hypotheses. Analysis of unpublished data from partial mtDNA sequences (11) produced a phylogenetic hypothesis fully compatible with, but less resolved, than ours and an approximate divergence time of 46 Myr for the split of *Onychodactylus* from all remaining hynobiid taxa (they used an absolute molecular clock calibration of 0.65% change per lineage per Myr, contrasted with our estimate of ≈110 Myr; Fig. 3). Their other estimates for times are correspondingly shorter as well. Our analysis included samples of all major lineages of hynobiids; most missing taxa are members of the deeply nested *Hynobius* and are unlikely to change any of our major results. Hynobiidae is an ancient lineage that has largely remained close to its likely site of origination, with the Central and Western Asian *Paradactylodon* and *Ranodon* being the primary outliers. The expansion of the range of *Salamandrella* to the west (it now occurs from Hokkaido to east of the Ural Mountains) is likely a very recent, post-Pleistocene event. Salamander evolution is characterized by extensive homoplasy, as documented herein and elsewhere (33, 34), and the growing molecular databases will be critically important in generating robust phylogenetic hypotheses for the still unresolved portions of caudate phylogeny (35, 36). Finally, as a model system of vicariance, further research on living salamanders will help clarify the role of Earth history in establishing biogeographic patterns and lineage diversification.

## Materials and Methods

**Taxonomic Sampling.** Currently, nine genera (and 50 species) of hynobiids are recognized: *Batrachuperus*, *Hynobius*, *Liua*, *Onychodactylus*, *Pachyhynobius*, *Pseudohynobius*, *Ranodon*, *Salamandrella*, and *Protohynobius* (see http://amphibiaweb.org). Our samples include eight genera and more than three species in some polytypic genera (*Hynobius* and *Batrachuperus*). For *Pseudohynobius*, we selected *P. tsinpaensis*, but recent work by Zeng *et al.* (37) transfers this species to *Liua*, at the same time showing that *Liua* is the closest relative of *Pseudohynobius*, so this change is unlikely to affect our results. We are unable to include the recently described *Protohynobius* (38), known only from a long-preserved single specimen. For outgroups we selected two other Chinese salamanders, the giant salamander *Andrias davidianus*, a member of the Cryptobranchidae thought to be the sister taxon of the Hynobiidae, and *Paramesotriton hongkongensis* (Salamandridae), which represents a more remote outgroup; complete mtDNA sequence exists for both taxa (31, 39). All species used in this study are shown in Table 1 along with their DNA Data Base in Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank accession numbers.

**Sequence Data Preparation.** Genomic DNA was purified from ethanol-preserved samples by using the UNIQ-10 genomic DNA extraction kit (Sangon, Shanghai, China) following the manufacturer's protocol. The circular mitochondrial DNA was amplified in two overlapping fragments >5 kb (to avoid amplifying nuclear

copies) by using both universal and specific primers (primer sequences are available from the authors upon request). Methods for amplification and sequencing are given elsewhere (see refs. 39 and 40 for details). Multiple alignments were prepared for two rRNAs, 22 tRNAs, and 12 protein-coding genes (ND6 was excluded) by using CLUSTAL X (41) at default settings. To avoid bias in refining alignments, ambiguous alignment positions (including tRNA loops, beginnings and ends of many protein-coding genes, and rRNA highly variable regions) were excluded by using the GBLOCKS program (42) at default settings.

**Phylogenetic Analyses.** DNA molecular phylogenies were reconstructed by using MP, NJ, Bayesian inference of likelihood (BA), and ML methodologies. ML was implemented by using PAUP* (Version 4.0b8) (43) with TBR branch swapping (10 random addition sequences). The best-fitting nucleotide substitution model was selected by using MODELTEST (Version 3.06) (44). MP was implemented in PAUP* by using heuristic searches (TBR branch swapping; MULPARS option in effect) with 10 random-additions sequences; all positions were given equal weights. NJ analyses were based on ML distance matrices taking account of the heterogeneity of rates among sites with a discrete gamma distribution. Nonparametric bootstrap support for internal branches was calculated for both MP and NJ with 1,000 replicates. Bayesian inference of likelihood was implemented by using MRBAYES (Version 3.1) (45) with both nonpartitioned and partitioned strategies. Under the partitioning strategy, the data set was divided into 15 partitions: two rRNAs, the concatenated tRNAs, and 12 protein-coding genes. The best-fitting nucleotide substitution models for each of the 15 partitions and the unpartitioned data set were selected by using the hierarchical likelihood ratio test implemented in MRMODELTEST (Version 1.1b) (46). Flatirichlet distributions were used for substitution rates and base frequencies, and default flat prior distributions were used for all other parameters. Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses (with random starting trees) were run with one cold and three heated chains (temperature set to default 0.2) for 1 million generations and sampled every 100 generations. Bayesian posterior probabilities were then calculated from the sample points after the MCMC algorithm started to converge. To ensure that our analyses were not trapped in local optima, three independent MCMC runs were performed. Topologies and posterior clade probabilities from different runs were compared for congruence. Ancestral state reconstruction was performed by using MRBAYES (Version 3.1) (1,000,000 generations).

Our analysis is based on extensive sequences of a single, uniparental marker. Despite possible negative consequences (47), we think that complete mitochondrial genomes are appropriate for the phylogenetic level of our study. At deeper phylogenetic levels, such as in our study, there is little concern with such problems as lineage sorting and mitochondrial introgression, and complete mitochondrial genomes are appropriately used as phylogenetic estimators.

**Molecular Divergence Estimates.** To test the validity of a molecular clock, a likelihood ratio test was used to test deviations from clock-like evolution. Date estimates for uncalibrated nodes in phylogenies were derived by using R8S (18) (Version 1.5; M. J. Sanderson, http://ginger.ucdavis.edu/r8s) with two algorithms: Langley–Fitch (local molecular clock) and penalized-likelihood. The ML tree was used as the required phylogram. The ingroup root was fixed to 161 $\pm$ 5 Myr based on the recent palaeontological findings of earliest known cryptobranchoids from the late Jurassic ($\approx$161 Mya) (6). The smoothing parameter in the penalized-likelihood analysis was selected by using the cross-validation test (18). Confidence intervals for divergence dates are based on the curvature of the likelihood surface as implemented in R8S.

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