Spiny frogs (Paini) illuminate the history of the Himalayan region and Southeast Asia

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Asian frogs of the tribe Paini (Anura: Dicroglossidae) range across several first-order tectono-morphological domains of the Cenozoic Indo-Asian collision that include the Tibetan Plateau, the Himalayas, and Indochina. We show how the tectonic events induced by the Indo-Asian collision affected the regional biota and, in turn, how the geological history of the earth can be viewed from a biological perspective. Our analysis of a concatenated dataset comprising four nuclear gene sequences of Paini revealed two main radiations, corresponding to the genera Nanorana (I) and Quasipaa (II). Five distinct clades are recognized: Tibetan plateau clade (I-1), Himalaya clade (I-2), environs of Himalaya–Tibetan plateau clade (I-3), South China clade (II-1), and Indochina clade (II-2). This pattern of relationships highlights the significance of shaping evolutionary history. Building on our molecular dating, ancestral region reconstruction, and distributional patterns, we hypothesize a distinct geographic and climatic transition in Asia beginning in the Oligocene and intensifying in the Miocene; this stimulated rapid diversification of Paini. Vicariance explains species formation among major lineages within Nanorana. Dispersal, in contrast, plays an important role among Quasipaa, with the southern Chinese taxa originating from Indochina. Our results support the tectonic hypothesis that an uplift in the Himalaya–Tibetan plateau region resulting from crustal thickening and lateral extrusion of Indochina occurred synchronously during the transition between Oligocene and Miocene in reaction to the Indo-Asian collision. The phylogenetic history of Paini illuminates critical aspects of the timing of geological events responsible for the current geography of Southeast Asia.

China | nuclear DNA | phylogeography | Tibet | tribe Paini

The collision between India and Asia may be the largest active orogenic event on earth. Following the initial collision in the early Cenozoic (50–55 Mya) or even earlier, about 70 Mya (1), many effects associated with the major tectonic episode continued through the Oligocene and well into the Miocene (2). During these times, associated geological processes occurred, ranging from the uplift (thickening) of the Himalaya–Tibetan plateau to lateral extrusion of the continental landmass (2,3) (Fig. S1). However, details of spatiotemporal evolution related to creation of the Himalayas and the Tibetan Plateau are still debated (1,2,4,5). This first-order tectonic feature is of great interest to biologists and geologists alike. The associated orogenic and environmental effects (e.g., geomorphology, climate change) likely served as a major driving force for modifying and influencing genetic discontinuities, speciation, and evolution of organisms. The geological dynamics both reduced physical barriers to range expansion and formed new barriers that promoted variability. We expect the evolutionary history of organisms in this region to parallel geological and related climate development, and even to suggest unrecorded earth historical events.

 Amphibians are ideal organisms for inferring geological and environmental history (6–8). The amphibian evolutionary tree, largely based on molecular data, is being revealed at a rapid pace. However, the evolutionary history of one major group—spiny frogs belonging to the tribe Paini, ranoid frogs endemic to Asia—is not well understood, and its classification remains unsettled (9–13). Dicroglossine ranoids, including Paini, form a lineage thought to have originated in present-day India (14). Adults or tadpoles of the tribe Paini (stone frog is the common name in China) live mostly in swift boulder-strewn streams in the mountains of South and Southeast Asia across the Himalayas, Indochina, and southern China (15). The specific habitat requirements suggest that these species are poor overland dispersers and likely were affected by outcomes associated with large-scale crustal deformation (i.e., >100 km). Their current distribution appears to be closely related to specific tectonomorphological features, including the Tibetan Plateau, the Himalayas, the Hengduan Mountain Range, and Indochina, which include three biodiversity hotspots (16). These findings raise questions of when, where, and how these frogs evolved. To answer these questions, a robust phylogeny is needed, together with estimates of divergence times of living Paini, which also should provide clues concerning the effects of geological events and enable testing geological hypotheses related to tectonic evolution in Asia.

Here, we present a comprehensive molecular phylogeny for members of Paini based on four nuclear DNA loci. We used several combinations of calibration points to obtain conservative timing estimates for the major early diversification events of this group, combined with ancestral area reconstruction. As we will show, the tribe Paini is a good biological model system for generating and testing hypotheses of the spatiotemporal history and significance of tectonic events in Asia.

Results

Sequence Characteristics. DNA sequences were obtained from four nuclear fragments for 36 specimens: 521 bp of tyrosinase, 315 bp of rhodopsin, 1,269 bp of Rag-1, and 1,143 bp of Rag-2 (Table S1). Almost all PCR and sequence reactions were successful, with the exception of Rag-2 for Hoplobatrachus rugulosus. The alignment of Rag-2 revealed some gap regions. Fejervarya limnocharis and Fejervarya cancrivora both have a 3-bp deletion region. Nanorana parkeri has a 15-bp deletion region, and Nanorana pleskei and Nanorana ventripunctata both have a 9-bp deletion region. The final combined DNA alignment is fairly unambiguous; it contains 3,248 nucleotide sites, 885 of which were variable and 554 of which were potentially phylogenetically informative. All sequences were deposited in GenBank (Table S2). All sequences were translated into amino acids without stop codons.


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Data deposition: The sequences reported in this paper have been deposited in the GenBank database. For a list of accession numbers, see Table S2.

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**Phylogenetic Analysis.** Initially, we intended to analyze each nuclear gene separately, but the limited information content of each gene prevented us from obtaining robust results. Accordingly, we combined the four nuclear genes and used Bayesian inference (BI) with a partitioned strategy. Bayesian analyses supported the monophyly of the tribe Paini (Fig. 1A). Within the tribe, two major clades (I and II) were recovered, with five well-supported monophyletic subclades (I-1, Tibetan plateau clade; I-2, Himalaya clade; I-3, environs of Himalaya–Tibetan plateau clade; II-1, South China clade; and II-2, Indochina clade), each defined largely by geography. Within clade I, Tibetan plateau taxa (I-1) and Himalaya taxa (I-2) are sister lineages, which, together, are sisters to clade I-3. Within clade II, an Indochina group (II-2) is sister to all other species (II-1), including another Indochinese clade (II-2-2) and the taxa exclusively found in South China (II-2-1).

**Temporal and Spatial Diversification. Estimation of divergence times.** The estimated divergence times for Paini are visualized in Fig. 2, and the divergence time estimates for the in-group nodes (Fig. S2) are shown in Table S3. The initial divergences among Paini were about 27 Mya [95% credibility interval (CI): 19–36 Mya]. Within group I, the Himalaya–Tibetan plateau taxa (I-1 and I-2) diverged from environs of the Himalaya–Tibetan plateau clade (I-3) ≈23 Mya (95% CI: 15–31 Mya). The split between the clade including Himalaya taxa (I-2) and the Tibetan plateau clade (I-1) is dated at ≈19 Mya. The diversification of group II occurred about 24 Mya (95% CI: 16–32 Mya). The second Indochinese clade (II-1-2) diverged from the clade of southern Chinese taxa (II-1-1) about 18 Mya.

**Biogeographic reconstructions.** The overall log-likelihood for our Lagrange analysis was −74.9 with the use of values of λD = 0.006 and λE = 0.003. The most probable ancestral area and the corresponding likelihood estimate values from Lagrange analysis for major nodes are shown in Fig. 2, together with the most probable ancestral area and posterior probability values of ancestral area reconstructions from Bayes dispersal-vicariance analysis (DIVA). For the Lagrange analyses, although some ambiguity and possible alternative resolutions exist, the highest likelihood estimates were consistent with the result of Bayes-DIVA. We considered it most likely for the hypotheses here.

The Tribe Paini originated from the Indochinese region (Fig. 2; 0.72 and 0.98 for the likelihood estimate value from Lagrange analysis and posterior probability value from Bayes-DIVA, respectively). The most recent common ancestor had likely already
spread in Indochina and in some western regions of China, although support is low (0.49 and 0.35 for the likelihood estimate value from Lagrange analysis and posterior probability value from Bayes-DIVA, respectively). A subsequent vicariant event separated two lineages of ancestral Painsi, giving rise to Nanorana in the general area of western China and Quasipaa in Indochina. Within Quasipaa, an important dispersal event spread the clade from Indochina to South China and, subsequently, a vicariant event isolated clades II-1 and II-1-2 within the subgenus Quasipaa (clade II-1, 0.50 and 0.51 for the likelihood estimate value from Lagrange analysis and posterior probability value from Bayes-DIVA, respectively). Within Nanorana, a vicariant event would explain the separation of the subgenus Chaparana (I-3) from the subgenera Nanorana and Paa (I-1 + I-2) (0.44 and 0.58 for the likelihood estimate value from Lagrange analysis and posterior probability value from Bayes-DIVA, respectively). The common ancestor of the subgenera Paa and Nanorana occurred in the region of the Himalaya–Tibetan plateau (class I terrain) (0.49 and 0.98 for the likelihood estimate value from Lagrange analysis and posterior probability value from Bayes-DIVA, respectively), and the recent ancestor of the subgenus Chaparana mainly occurred outside of the Himalaya–Tibetan plateau region (class II terrain).

Discussion

Origin, Diversification, and Trait Evolution of the Tribe Paini. The discovery of two major groups with five distinct lineages that have geographic identity (SI Text) was unexpected. Previous studies have been hampered by a complicated taxonomic history and unresolved relationships (9–11).

Our molecular dating and ancestral area reconstruction analyses (Fig. 2) indicate that the tribe Paini originated from present-day Indochina. The common ancestors of the tribe likely had spread into adjacent western China by the start of the Oligocene (≈27 Mya). The clade rapidly diversified through the Oligocene into the Miocene (Fig. 3) as Asia transitioned from a zonal pattern to a monsoon-dominated pattern, which was a significant climatic shift (17). At this time, the general western China group (Nanorana) and the Indochina group (Quasipaa) split, based on our ancestral area inference. Modern taxa of Nanorana are mostly distributed in West China at relatively higher elevations than the more lowland species of Quasipaa (Fig. 3B).

Nanorana. Vicariance played a significant role in the diversification of Nanorana. Dramatic crustal deformation induced by the Indo-Asian collision contributed to the complicated landscapes in western China. Massive mountains and deeply carved valleys existed, which acted as barriers to distribution and resulted in species formation, initially between lineages, that subsequently gave rise to the subgenera Nanorana, Paa, and Chaparana at about 23 Mya (Fig. 2). Taxa from the Himalayas and Tibetan plateau (class I terrain) became isolated from peripheral regions (class II terrain). Later, continued uplift of the Himalayan region (2) finally cut off genetic exchange between the Himalayan range and the interior Tibetan plateau, resulting in the split between the subgenera Nanorana and Paa at about 19 Mya. Further diversification produced additional species within the subgenus Nanorana in the Tibetan plateau region at about 9 Mya, by which time the plateau had likely reached significant altitude, with extreme environments prevailing in the interior (e.g., on the more or less flat Tibetan plateau with numer-called Tibetan plateau with numerical identifications).
ous lakes but with an arid to semiarid climate and a cold atmosphere) (Fig. 1D).

Compared with what we consider to be typical members of the tribe Paini (i.e., stream-adapted, large body size; Fig. 1B, C, and E), the three species on the Tibetan plateau are characterized by life in still water and relatively small body size (Fig. 1D), as well as by having a reduced (or even absent) tympanum and columella (implying some loss of auditory function) (13). Ancestral state reconstruction (stream vs. still water) suggests that Paini originated from a stream-adapted ancestor and then invaded still water in response to drastic environment changes on the Tibetan plateau. Organ degeneration probably is in response to environmental extremes (e.g., low temperature, persistent wind, arid to semiarid climate, hypoxia, food shortage with increasing altitude) (18). For example, low oxygen levels on the Tibetan Plateau are not suitable for many organisms; a high level of calling during courtship is metabolically demanding (19). Nontympanic pathways of sound reception and ultrasonic signal system may be used to compensate for anuran acoustic organ degeneration (20), but frogs of the subgenus *Nanorana* need more study.

**Quasipaa.** The southern Chinese clade, subgenus *Quasipaa* (II-1-1) and the Indochinese taxa of the subgenus *Quasipaa* (II-1-2) were recovered as more closely related than the two Indochinese taxa clades (II-1-2 and II-2) are to each other. The southern Chinese taxa (II-1-1) (Fig. 1A) are deeply nested within those Indochinese groups (II-2, II-1-2); this suggests that Indochina was the place of origin for the southern Chinese taxa. Historical biogeographic analysis supports this hypothesis (Fig. 2). The common ancestor of *Quasipaa* was likely distributed in present-day Indochina, and dispersal from Indochina to South China likely occurred between 24 and 18 Mya. The split of the genus *Quasipaa* into the current subgenera *Quasipaa* (II-1) and *Eripaa* (II-2), initiated about 24 Mya, is probably linked to orogenic movement of the Truong Son Mountain Range (Fig. 3) along the border between Laos and Vietnam, although relevant geological studies of this region are few. The Truong Son Mountain Range forms a distinct divide between the relatively dry Thai-Lao Plateau (highland) of central Indochina and the lowlands of Vietnam to the east. The complete isolation between the Indochinese (II-1-2) and exclusive southern Chinese (II-1-1) taxa occurred after about 18 Mya. The South China Sea probably opened in the
Mioocene, and this event finally led to a barrier between South China and Indochina (21). Ductile movements of the Red River zone caused the extrusion of Indochina and the offshore rift between Indochina and South China; these events provide clues to explain the present distributional pattern of Quasipaa. The presence of endemic species of clade II-1-2 adds a unique component to the central part of the Truong Son Mountain Range, as evidenced also by some Insectivora (22). The close relationship between these Indochemical taxa (clade II-1-2) and the southern Chinese taxa (clade II-1-1) suggests that the divergence of clade II-1-2 offers important clues to explain the origin of the South China fauna. Central Vietnam likely served as a refugium for forest specialists during geological movements and climatic oscillations. These findings highlight the conservation priorities and protected area designation of this region (23).

Most species of Paini breed in streams, and, characteristically, male frogs have clusters of spines, secondary sexual characters used during amplexus, on the chest or more or less scattered on the belly. Ancestral state reconstruction supports three independent secondary losses of spines on the belly (Fig. 1A). Within the subgenus Chaparana, this loss happened twice, first in Nanorana talhangnica and Nanorana quadramus and then later in Nanorana unculuanus. In the subgenus Quasipaa, this loss occurred only once (Quasipaa yei). These four species also lack hypertrophied forelimbs and have fingers without spines during the breeding season, suggesting different evolutionary origins compared with other Paini species. Generally, our data support the hypothesis that during the breeding season, nuptial spines always are accompanied by an enlarged forearm as well as spines on fingers (24).

Implications for the Geological Evolution of Asia. Except for the subgenus Nanorana, most species of the tribe Paini prefer to sit on moss-covered rocks near cold mountain streams. This long-lived conservation life trait, coupled with their present distribution in Asia, makes this species group a potential model for examining geological evolution and environmental changes in Asia during the Tertiary.

Since the late Oligocene, uplift of the general western Chinese area, including the present Himalayas, the Tibetan plateau, and their environs (class I and II terrain; Fig. S3), led to the high western (class I + II) and low eastern (class II) terrains, which have subsequently characterized the topography of China. The associated geological events (Fig. 2A) probably also triggered climate change, which, in turn, led to biotic reorganization in Asia. Our molecular dating results reflect this event. The tribe Paini began diversification during the Oligocene, and rapid divergences of the major clades took place in the Miocene (Fig. 2), suggesting geographical and probably paleoclimatic change. This is consistent with inferences from mammalian fossil data, paleobotanical evidence, and lithological and sedimentary data (17, 25, 26). The paleobotanical and lithological evidence shows radical changes in climate and vegetation from the Oligocene to the Miocene (transition between the Paleogene and Neogene) (26). Furthermore, since Oligocene time, mammalian faunal turnovers have occurred in northwestern China, and significant reorganization of faunas followed during the Miocene (25, 27).

The split between the subgenera Paa, Nanorana (clades I-1 + I-2), and Chaparana (clade I-3) (Fig. 2B: ≈23 Mya) suggests continuous uplift of the Himalayas and Tibetan plateau system, which caused the separation between the Himalaya–Tibetan plateau (class I terrain) and its environs (class II terrain). At about the same time (≈24 Mya), we infer the split of the subgenus Quasipaa (clade II-2) and Eripa (clade II-1) associated with a major tectonic event along the Truong Son Mountain Range, which served to divide the western highlands and eastern lowlands along the Ailao Shan-Red River shear zone. The shear zone continued along the coast of Vietnam far to the south (Fig. S1), and its motion may have created the modern Truong Son Mountain Range and the opening of the South China Sea. Our dating is broadly consistent with the geological hypothesis that collision of India with Asia displaced Indochina southeastward relative to South China along the Ailao Shan-Red River shear zone, which occurred mostly in the Oligocene and early Miocene (3). The distribution of the genus Quasipaa (clade II), with isolation of the subgenus Quasipaa (South China block) from Eripa (Indochina block), may reflect this event. The relative importance of crustal thickening vs. lateral extrusion attributable to the Indo-Asian collision has been intensely debated (4, 28). Our study suggests that the geologically hypothesized uplift (thickening) and strike-slip extrusion must have occurred simultaneously to generate the observed biotic distribution pattern at the Oligocene–Miocene boundary (≈23–24 Mya) (Fig. 2B).

The split of the subgenera Nanorana (clade I-1) and Paa (clade I-2) took place about 19 Mya, associated with geological events that separated the Himalayan region and the Tibetan plateau (Fig. 2C). This result is compatible with other geological hypotheses that possible rapid uplift of southern Tibet and the Himalayas began about 20 Mya (2). The diversification of the subgenus Nanorana on the Tibetan plateau took place about 9 Mya (Fig. 2), which suggests that species formation may be associated with active orogeny within Tibet; this hypothesis is compatible with the geological hypothesis that rapid uplift of Tibet took place about 8 Mya (2, 29, 30). All three species of the subgenus Nanorana dwell at altitudes from nearly 3,000 m up to 4,700 m (Fig. 3B). Members of this subgenus adapted progressively as the entire Tibetan plateau experienced significant increases in altitude to at least the 3,000-m level (Fig. 2D).

In short, our data and analyses bring large-scale features of Asian Tertiary history and topography into a framework consistent with the evolutionary history of the tribe Paini. Multistage uplift events in different regions (Fig. 2) and a strike-slip extrusion event affecting Indochina (Fig. 2B) fit well with our conception of frog biogeography. Analyses of other taxa will provide tests of our hypotheses.

Materials and Methods

Taxon Sampling. Species of Paini, genera Nanorana and Quasipaa, formed our in-group, and species in the genera Hoplobatrachus, Limnonectes, and Fejervarya were used as out-group taxa. Thirty-six specimens in total were used, among which 29 individuals representing at least 24 species formed the in-group. All sampled in-group species and locations are given in Table S2 and Fig. 3. Our sampling included nearly all recognized species representing the five major clades recovered (10) using a bottom-up approach (31). We focused on resolving the basal relationship with large numbers of characters using relatively slowly evolving nuclear genes.

Extraction, Amplification, and Sequencing. Muscle or liver tissue samples were stored in 95% or 100% ethanol or were frozen at −80 °C. DNA was extracted using the standard three-step phenol/chloroform extractions (32). Primers used in PCR and sequencing of the four nuclear protein-coding gene fragments are shown in Table S4. Amplification was performed in a 25- to 50-μL volume reaction using the same procedures as other studies (10, 33). Purified PCR products were directly sequenced with an automated DNA sequencer ABI 3730 (Applied Biosystems, Foster City, CA) in both directions for each species. They were submitted for a BLAST search in GenBank and also translated into amino acids to ensure the target sequences had been amplified.

Data Analysis. All the nuclear sequence data derived from the genes for rhodopsin, tyrosinase, Rag-1, and Rag-2 were easily aligned with Clustal X 1.81 (34) with default parameters and then verified by eye using Molecular Evolutionary Genetics Analysis (MEGA) 4 (35). Reconstructions of phylogenies were primarily performed using BI (36) with a partitioned strategy. Details are given in SI Materials and Methods.

Temporal and Spatial Diversification. Divergence time estimates. We estimated nodal ages within the tribe Paini and with 95% Cs from our nuclear DNA sequence data using the Bayesian molecular clock method implemented in the software BEAST version 1.4.7 (37). We used two indirect estimates of divergence time as calibration points (38), which is similar to the method used in
another study (39). The two studies (38, 39) included groups having more extensive fossil records. Furthermore, we also used an additional calibration point from biogeographic inference (40). Three calibration points were used in total, and details are given in SI Materials and Methods.

Ancestral area reconstructions. Biogeographic reconstructions were performed using Bayes-DIVA (41) and Lagrange 2.0 based on the stochastic model of geographic range evolution (42, 43). Details are given in SI Materials and Methods.

Ancestral state estimation. Ancestral values of the male secondary characteristics were estimated using maximum likelihood and maximum parsimony in the program Mesquite (44). Most species can be clearly coded as having the spines on the breast or ventral region, fingers with spines, and hypertrophied forelimbs during the breeding season, except for *N. quadrans*, *N. taihangnica*, *N. unculuanus*, and Q. yei (13). Furthermore, most species of Paini can be coded as stream dwellers, and only the three species of the subgenus *Nanorana* belong to the still-water dwellers. Overall, parsimony and likelihood gave the same results.

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