



# Relict groups of spiny frogs indicate Late Paleogene-Early Neogene trans-Tibet dispersal of thermophile faunal elements

Sylvia Hofmann<sup>1</sup>, Daniel Jablonski<sup>2</sup>, Spartak N. Litvinchuk<sup>3</sup>, Rafaqat Masroor<sup>4</sup> and Joachim Schmidt<sup>5</sup>

<sup>1</sup> Centre of Taxonomy and Evolutionary Research, Zoological Research Museum Alexander Koenig, Bonn, Germany

<sup>2</sup> Department of Zoology, Comenius University in Bratislava, Bratislava, Slovakia

<sup>3</sup> Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia

<sup>4</sup> Zoological Sciences Division, Pakistan Museum of Natural History, Islamabad, Pakistan

<sup>5</sup> Institute of Biosciences, General and Systematic Zoology, University of Rostock, Rostock, Germany

## ABSTRACT

**Background.** The Himalaya-Tibet orogen (HTO) presents an outstanding geologically active formation that contributed to, and fostered, modern Asian biodiversity. However, our concepts of the historical biogeography of its biota are far from conclusive, as are uplift scenarios for the different parts of the HTO. Here, we revisited our previously published data set of the tribe Pains extending it with sequence data from the most western Himalayan spiny frogs *Allopaa* and *Chrysopaa* and using them as an indirect indicator for the potential paleoecological development of Tibet.

**Methods.** We obtained sequence data of two mitochondrial loci (16S rRNA, COI) and one nuclear marker (Rag1) from *Allopaa* samples from Kashmir Himalaya as well as *Chrysopaa* sequence data from the Hindu Kush available from GenBank to complement our previous data set. A Maximum likelihood and dated Bayesian gene tree were generated based on the concatenated data set. To resolve the inconsistent placement of *Allopaa*, we performed different topology tests.

**Results.** Consistent with previous results, the Southeast Asian genus *Quasipaa* is sister to all other spiny frogs. The results further reveal a basal placement of *Chrysopaa* relative to *Allopaa* and *Nanorana* with an estimated age of *ca.* 26 Mya. Based on the topology tests, the phylogenetic position of *Allopaa* as a sister clade to *Chaparana* seems to be most likely, resulting in a paraphyletic genus *Nanorana* and a separation from the latter clade around 20 Mya, although a basal position of *Allopaa* to the genus *Nanorana* cannot be entirely excluded. Both, the placements of *Chrysopaa* and *Allopaa* support the presence of basal Pains lineages in the far northwestern part of the HTO, which is diametrically opposite end of the HTO with respect to the ancestral area of spiny frogs in Southeast Asia. These striking distributional patterns can be most parsimoniously explained by trans-Tibet dispersal during the late Oligocene (subtropical *Chrysopaa*) respectively early Miocene (warm temperate *Allopaa*). Within spiny frogs, only members of the monophyletic *Nanorana*+*Paa* clade are adapted to the colder temperate climates, indicating that high-altitude environments did not dominate in the HTO before *ca.* 15 Mya. Our results are consistent with fossil records suggesting that large parts of Tibet were characterized by subtropical to warm temperate climates at least until the

Submitted 19 February 2021

Accepted 25 June 2021

Published 15 July 2021

Corresponding author

Sylvia Hofmann,  
s.hofmann@leibniz-zfmk.de

Academic editor

Diogo Provete

Additional Information and  
Declarations can be found on  
page 14

DOI 10.7717/peerj.11793

© Copyright

2021 Hofmann et al.

Distributed under

Creative Commons CC-BY 4.0

OPEN ACCESS

early Miocene. They contradict prevalent geological models of a highly uplifted late Paleogene proto-Plateau.

**Subjects** Biodiversity, Evolutionary Studies, Molecular Biology, Taxonomy, Zoology

**Keywords** Phylogenetic, Paini, Himalaya, Biogeography, Paleogene, *Allopaia*, *Chrysopaa*, Tibet

## INTRODUCTION

The uplift of the modern Himalaya-Tibet orogen (HTO) was one of the most extensive geological events during the Cenozoic. Today's dimension of the HTO is thought to exert profound influences on the regional and global climate, and, consequently, on Asian biodiversity. Thus, understanding the evolution and knowing the past topography of the HTO is critical for exploring its paleoenvironments and historical biogeography (*Kutzbach et al., 1989; Molnar, Boos & Battasti, 2010; Raymo & Ruddiman, 1992; Zhang et al., 2018*). However, various lines of geoscientific evidence have suggested—partly substantially—different uplift scenarios for the respective parts of the HTO (reviewed in *Spicer et al., 2020*). These scenarios range from the idea of a simple monolithic rising of Tibet purely due to crustal thickening or lithosphere modification (e.g., *Wang et al., 2014; Zhao & Morgan, 1985*), over different models of a fractional, stepwise development (e.g., *Tapponnier et al., 2001*), to the concept of a high 'proto-Tibetan Plateau' (*Mulch & Chamberlain, 2006; Wang et al., 2014*). Linked to these varying conceptions are uncertainties in timing, quantity (elevational increase) and sequence pattern of the HTO uplift. While several geoscientific studies present evidence for a high elevated Tibetan Plateau (TP) as early as the Eocene or even earlier (e.g., *Kapp et al., 2007; Murphy et al., 1997; Tapponnier et al., 2001; Wang et al., 2008; Wang et al., 2014*) others assume elevations close to modern values by the latest at the middle Oligocene (*Ding et al., 2014; Quade et al., 2011; Rowley & Currie, 2006; Xu et al., 2013*) or that a massive uplift occurred in the late Neogene (e.g., *Molnar, England & Martiod, 1993; Su et al., 2019; Wei et al., 2016*).

During the last decade, a growing number of paleontological studies provide evidence for low elevated parts of Tibet until the early Neogene or even later; for example, the presence of subtropical to warm temperate floras during the late Eocene to early Miocene have been demonstrated for the basins of Hoh Xil, Kailas, Lunpola, Nima, and Qiabulin of southern and central parts of the Plateau (*Ai et al., 2019; Ding et al., 2020; Miao et al., 2016; Su et al., 2019; Sun et al., 2014; Wu et al., 2017*). These findings suggest that the present high-plateau character of Tibet with its dominant alpine environments is apparently a recent formation that did not emerge before the mid-Miocene. The young ages of species divergence in the phylogenies of high-altitude taxa endemic to the plateau are a logical consequence of—and evidence for—rather recent evolution of the TP (summary in *Renner, 2016; Hofmann et al., 2017; Hofmann et al., 2019*). However, although it is becoming increasingly acknowledged that the HTO contributed to, and fostered, modern Asian biodiversity (*Johansson et al., 2007; Steinbauer et al., 2016*), our present concepts of the origin and historic biogeography of the terrestrial biotas inhabiting the HTO are far from being complete nor conclusive

and have been hindered by a lack of and potential misinterpretation of data (Renner, 2016; Spicer, 2017; Spicer et al., 2020).

Phylogenies are a key mean in biogeographic and molecular evolutionary studies (Avice, 2009; Avice et al., 2000) and increasingly recognized as being essential to research that aim to reconcile biological and geological information to reconstruct Earth surface processes such as mountain building (Hoorn et al., 2013; Mulch & Chamberlain, 2018). In fact, organismal evolution offers an independent line of evidence for the emplacement of major topographical features, which have been proved valid in refining the timing of events substantiated by geologic record (Donoghue & Benton, 2007; Richardson et al., 2018). Specifically, several studies have demonstrated the suitability of phylogenetic data for addressing the timing and complexity of orogenic events, e.g., the Andean uplift and the formation of the Qinghai-Tibetan region (Antonelli et al., 2009; Luebert & Muller, 2015).

We here use spiny frogs of the tribe Paini (Dicroglossidae) to untangle the spatiotemporal evolution of this group in the HTO and, thus, as an indirect indicator for the topographic and paleoecological development of High Asia. Spiny frogs occur across the Himalayan mountain arc from northern Afghanistan, Pakistan, and northern India, through Nepal, Sikkim, and Bhutan, and in the valleys of southern and eastern Tibet, eastwards to eastern China, and southwards to the mountains of Indochina (Myanmar, Thailand, Laos, northern Vietnam; Frost, 2021). They live mostly in boulder-rich running water (Dubois, 1975) or clear pools with flowing water. Males are characterized by black, keratinous spines (Ohler & Dubois, 2006). The Paini tribe is currently composed of the genus *Nanorana* Günther, 1896 (around 30 species), *Quasipaa* Dubois, 1992 (11 species), *Allopaa* (Ohler & Dubois, 2006) (possibly two species), and the monotypic genus *Chrysopaa* (Ohler & Dubois, 2006). Following Che et al. (2010) and our own findings (Hofmann et al., 2019), *Nanorana* can be subdivided into three subgenera (*Nanorana*, *Paa*, and *Chaparana*). However, the phylogenetic and mostly taxonomic relationships among Paini are not completely resolved with several taxonomic changes during the last decade including taxa descriptions (Che et al., 2009; Frost, 2021; Huang et al., 2016; Jiang et al., 2005; Pyron & Wiens, 2011).

Previous studies proposed contrasting hypotheses to explain the current distributional and phylogenetic patterns of spiny frogs in the HTO. While a strict vicariance driven scenario suggests species formation among major lineages when the species were “trapped” in the mountain mass and become separated when it uplifted (Che et al., 2010), a more recent study found no clear support for this model but indications for a Paleo-Tibetan origin of Himalayan spiny frogs (Hofmann et al., 2019), confirming modern hypotheses for the past topographic surfaces of the southern parts of the HTO. This Tibetan-origin scenario (Schmidt et al., 2012) assumes that adaptation of Himalayan spiny frogs to the high-altitude environment occurred in South Tibet, at a time when the Greater Himalaya had not yet risen to its present height (Hofmann et al., 2019). With the continuously uplifting Himalaya along with the drying of southern Tibet, these ancestral lineages have probably been forced to follow the spatially shifted suitable habitats along the transverse river valleys of the Himalayas, such as the Brahmaputra, Kali Gandaki, or the Indus catchment (Hofmann et al., 2019). The hypothesis about the South-Tibetan origin has been also demonstrated

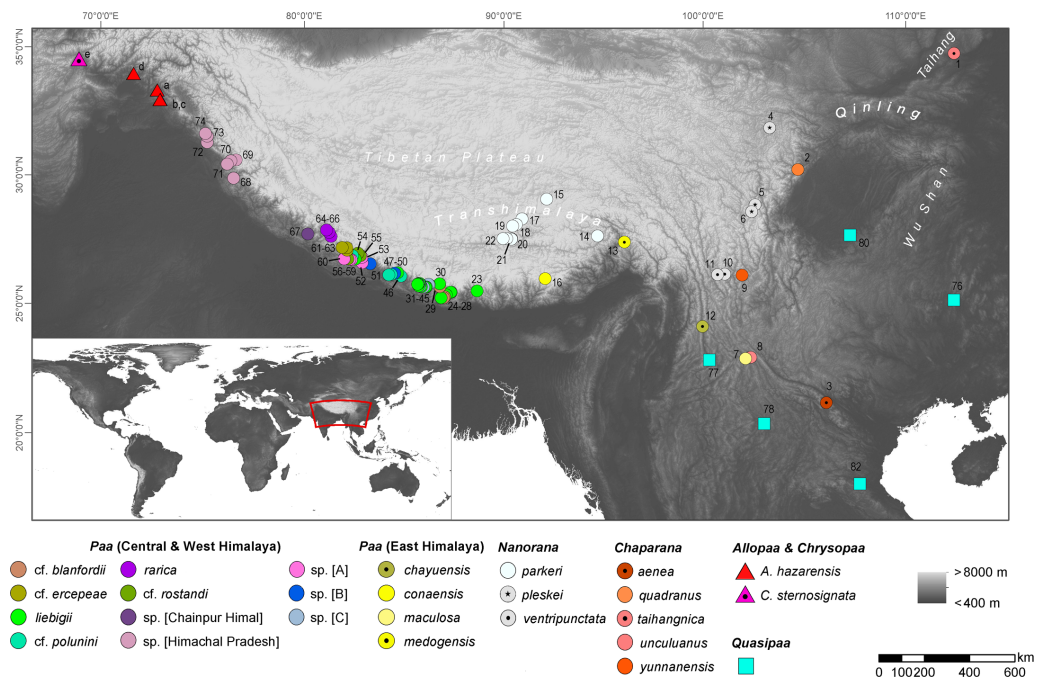
in other Himalayan faunal elements, e.g., *Scutigera* lazy toads ([Hofmann et al., 2017](#)) and forest-dwelling *Pterostichus* ground beetles ([Schmidt et al., 2012](#)).

So far, the phylogenetic placement of the westernmost Dicroglossid frogs that occur in the HTO (*Allopaa* from Kashmir Himalaya and *Chrysopaa* from Hindu Kush) has never been addressed. Given the Tibetan-origin hypothesis ([Hofmann et al., 2017](#); [Schmidt et al., 2012](#)), we expect that thermophile clades from the northwestern margin of the HTO represent distinct lineages and are not closely related to any of the geographically neighbouring lineages that occur in the Himalaya and on the central Tibetan Plateau. If so, a basal placement of these westernmost groups relative to other Himalayan Panni or a close relationship to thermophile taxa from the east of the Himalayan arc would argue for Paleocene dispersal of warm temperate or subtropical lineages westward to the northwestern margin of the HTO. Thus, integrating *Allopaa* and *Chrysopaa* into the analysis would allow better understanding of the time at which spiny frogs have adapted to high mountains and about which part of the paleo-HTO was occupied first by these amphibians. Consequently, the phylogeny of these frogs is of particular interest with respect to the controversial debate regarding the geological and paleoecological development of High Asia (see above). Therefore, we here reanalysed our previous dataset ([Hofmann et al., 2019](#)) by extending it with sequence data from *Allopaa* and *Chrysopaa*. We use our findings of the Panni phylogeny and time tree to discuss the biogeographic history of these frogs against the background of current HTO uplift concepts.

## MATERIALS & METHODS

### Sampling, laboratory protocols and data acquisition

We used sequence data of the 16S ribosomal RNA (rRNA), mitochondrial Cytochrome c oxidase I (COI) and nuclear Recombination activating gene 1 (Rag1) region available from our previous study ([Hofmann et al., 2019](#)) and complemented the data with a newly generated sequences for these three gene regions from *Allopaa hazarensis* ([Dubois, 1975](#)) ( $n = 6$ ; Pakistan, including the type locality of the species - Datta, Manshera District, Hazera Division; for details see [Fig. 1](#) and [Table S1](#)). Sampling was performed under the permit of the Pakistan Museum of Natural History, Islamabad, Pakistan (No. PMNH/EST-1(89)/05), according to the regulations for the protection of terrestrial wild animals. We also included 16S rRNA and COI sequence data of *Chrysopaa sternosignata* from Bagram, Parwan Province, Afghanistan (Hindu Kush Mts.) available in NCBI GenBank (accession numbers: [MG700155](#) and [MG699938](#)). Our *Nanorana* samples from Himachal Pradesh, which were previously referred to as “sp.” ([Hofmann et al., 2019](#)), were identified as *Nanorana vicina* based on morphological characters ([Boulenger, 1920](#); [Stoliczka, 1872](#)); for photos of live specimens [Fig. S1](#). Genomic DNA was isolated from ethanol tissues using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s protocol. Approximately 571 bp of the 16S, 539 bp of the COI, and a sequence segment of 1,207 bp of Rag1 gene were amplified using primers and PCR conditions as previously described ([Hofmann et al., 2019](#)). Amplicons were purified using the ExoSAP-IT enzymatic clean-up (USB Europe GmbH, Staufen, Germany) and the mi-PCR Purification Kit



**Figure 1** Map showing the origin of sequence data used in this study; locality numbers refer to samples and sequences listed in Table S1.

Full-size [DOI: 10.7717/peerj.11793/fig-1](https://doi.org/10.7717/peerj.11793/fig-1)

(Metabion, Planegg, Germany) or directly purified by Eurofins Genomics (Germany) with in-house protocols. The Sanger sequencing was performed on an ABI 3730 XL sequencer at Eurofins Genomics or by Macrogen Inc. (Seoul, South Korea or Amsterdam, The Netherlands; <http://www.macrogen.com>).

### Sequence alignment and phylogenetic reconstruction

We aligned our new 16S sequences to the previous secondary structures-based data set (Hofmann *et al.*, 2019) by eye; sequences of the protein-coding genes were aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA X (Kumar *et al.*, 2018). No ambiguities, such as deletions, insertions, or stop codons, were found neither in the alignment based on nucleotides nor in amino acids alignment.

The concatenated rRNA + mtDNA + nuDNA sequence alignment consisted of 184 taxa and contained 2,317 nucleotide positions of which 494 were phylogenetically informative. Nuclear data were unphased as most of the taxa were represented by only single individuals. We inferred a maximum-likelihood (ML) and a Bayesian inference (BI) tree based on the concatenated sequence data using RAXML v.8.2.12 (Stamatakis, 2014), IQ-TREE v.2.0 and MrBayes v.3.2.6 (Ronquist *et al.*, 2012). We partitioned the dataset a priori by gene and codon fragments and used PartitionFinder 1.1.1 (Lanfear *et al.*, 2012) to optimize the partition scheme with the following setting: branch lengths linked, corrected Akaike Information Criterion (AICc), greedy search algorithm, and the substitution models implemented in RAXML and MrBayes. RAXML was run with the GTRGAMMA model and

1,000 bootstrap replicates on the CIPRES (Cyberinfrastructure for Phylogenetic Research) (Miller, Pfeiffer & Schwartz, 2010). IQ-TREE was performed with the edge-linked partition model (Chernomor, von Haeseler & Minh, 2016) and both SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010) and the ultrafast bootstrap approximation (Hoang et al., 2018) using 1 Mio replicates per test. In the Bayesian analysis we assigned the doublet model (16×16) proposed by Schoniger & von Haeseler (1999) to the rRNA stem regions. Unambiguous stem pairs were inferred based on the consensus structure from RNAsalsa 0.8.1 (Stocsits et al., 2009) and implemented in the MrBayes input file. For the analysis of the remaining positions, the standard 4×4 option was applied using a GTR evolutionary model for all nucleotide partitions. The site-specific rates were set variable. For reasons of comparison, we also inferred the Bayesian tree using the 4×4 standard model of DNA substitution for all regions and the optimized models and partitions as suggested by PartitionFinder. MrBayes was run with a random starting tree for five million generations, sampling trees every 500th generation. Inspection of the standard deviation of split frequencies as well as an effective sample size value >200 of the traces using Tracer v. 1.7.1 (Rambaut et al., 2018) indicated convergence of Markov chains. In all analyses, we used four parallel Markov chain Monte Carlo simulations with four chains (three heated and one cold) and discarded the first 25% of the samples of each run as burn-in; consensus trees were produced using the sumt command.

To test competing topologies, we used a Bayes Factor (BF) approach and the tree topology tests implemented in IQ-TREE, namely the approximately unbiased (AU) test (Shimodaira, 2002) as well as the REL approximation (Kishino, Miyata & Hasegawa, 1990), including bootstrap proportion, Kishino-Hasegawa test (Kishino & Hasegawa, 1989), Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999), and expected likelihood weights (Strimmer & Rambaut, 2002). The marginal likelihoods estimations (MLE) for the BF calculations were obtained under each model based on both the stepping-stone (ss Xie et al., 2011) and path sampling (ps Lartillot & Philippe, 2006) methods implemented in BEAST v.1.10.4 (Suchard et al., 2018) using optimal partitions and substitution models as assessed in PartitionFinder, 250 million generations, a logging interval of 25,000, a MLE chain length of 1 million, and 100 path steps. Statistical support was then evaluated via 2lnBF using the ps/ss results as per Kass & Raftery (1995). Finally, we also used the stepping-stone approach with 10 million generations (4 runs and 4 chains), to estimate the model likelihood values for BF calculation with MrBayes by implementing the doublet option on 16S rRNA stem regions and the standard substitution option on all other regions. We specifically tested the hard constraint vs. negative constraint on *Chaparana* and *Allopaa*. In statistical hypothesis testing, models are compared to assess the strength of evidence against the null hypothesis ( $H_0$ ), which is defined as the one with the lower marginal likelihood (i.e., with the smaller value of the negative log-likelihood): 2lnBF <2 implies no evidence against  $H_0$ ; 2–6, weak evidence; 6–10, strong evidence; and >10 very strong evidence. For the REL approximation we used 1 Mio replicates, all other settings were left as default.

## Molecular dating

Divergence dates were estimated using BEAST2 v.2.6.2 (Bouckaert et al., 2014), based on the full concatenated dataset because of missing data in the alignment for some of the taxa (see Hofmann et al., 2019). Similar as to the MrBayes analyses, the partition scheme was optimized using PartitionFinder and the models that are implemented in BEAST. It is not possible to consider secondary structure information in BEAST (ambiguities are treated as unknown data so we did not remove stem regions)—thus all positions of the respective rRNA partition were treated under the same evolutionary model. Age constraints were derived from our previous calibration analysis of the phylogeny of *Nanorana*, which based on fossil-calibrated divergence estimates (Hofmann et al., 2019): MRCA of Paini 38.10 Ma, 28.70–47.50 (normal, sigma: 4.80); split of Tibetan *Nanorana* and Himalayan *Paa* 12.59 Ma, 7.93–17.30 (normal, sigma: 2.38); separation of the Plateau frog (*N. parkeri*) and *N. ventripunctata*+*N. pleskei* 6.35 Ma, 3.54–9.16 (normal, sigma: 1.44).

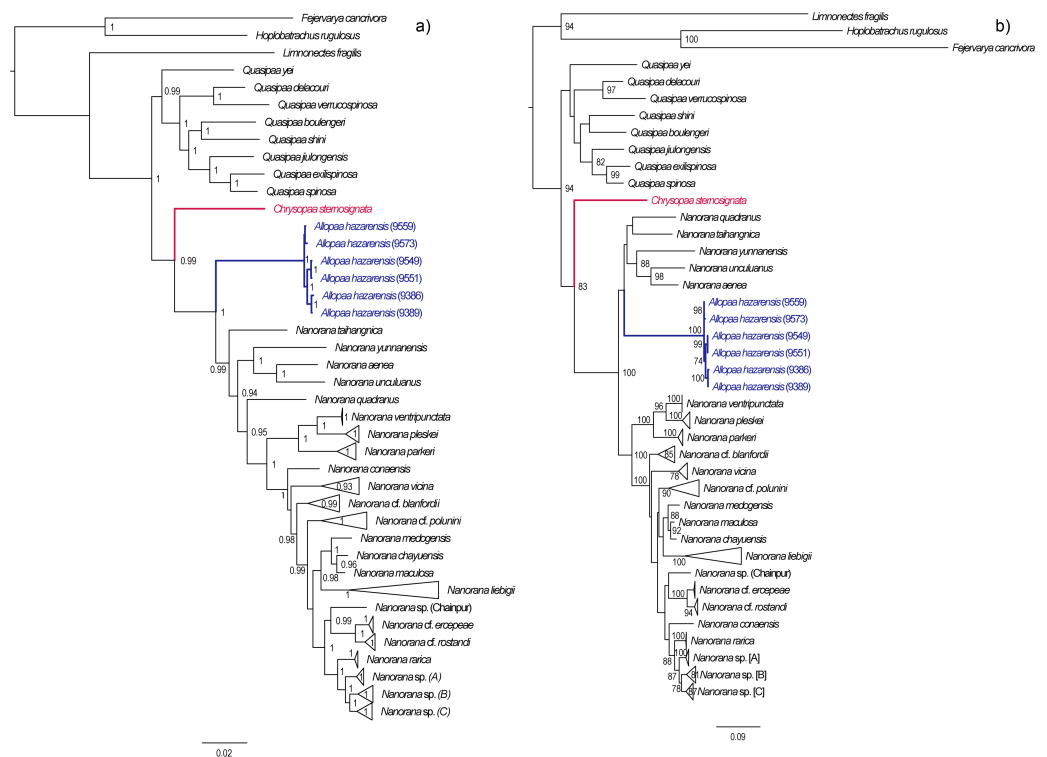
Analysis relied on ten independent BEAST runs with a chain length of 50 million, a thinning range of 5,000, a lognormal relaxed clock model, a Yule tree prior, a random starting tree, and the site models selected by bModelTest package (Bouckaert & Drummond, 2017) implemented in BEAST2. Runs were then combined with BEAST2 LogCombiner v.2.6.2 by resampling trees from the posterior distributions at a lower frequency, resulting in 9,010 trees. Stationary levels and convergence of the runs were verified with Tracer based on the average standard deviation of split frequencies and ESS values >200. The final tree was obtained with TreeAnnotator v.2.6.2 and visualized with FigTree v.1.4.3 (Drummond & Rambaut, 2007).

## RESULTS

### Phylogeny of Paini from the HTO

In both the ML and BI analyses, a relatively well resolved tree was obtained with strong support for most of the main clades, although with partly inconsistent and uncertain branching patterns of lineages within (sub)clades (Fig. 2). When information on secondary structure of 16S rRNA is considered (BI-tree), the results strongly support three monophyletic clades within Paini, apart from the monotypic *Chrysopaa: Quasipaa*, *Alloppaa*, and *Nanorana*, with *Alloppaa* forming the sister taxon to all *Nanorana*. Otherwise, *Alloppaa* clusters with *Chaparana*, which together form the sister clade to *Paa* and *Nanorana* subgenera in the ML-tree (see also Fig. S2 for topology generated with IQ-TREE and with MrBayes using the 4×4 substitution model, and Fig. S3 for ML trees based on 16S+COI and on Rag1 sequence data). The most striking result, consistently recovered in all trees, is the placement of *Chrysopaa* from the northern-central Afghanistan (Hindu Kush Mts.), which forms the sister taxon to *Alloppaa* and *Nanorana*.

In accordance with our previous findings, three monophyletic subclades can be distinguished within *Nanorana*, namely *Chaparana* from montane regions of the southeastern margin of the TP and mountains of NE China, *Paa* from high-montane regions of the West, Central and East Himalaya, and nominal *Nanorana* from subalpine and alpine regions of the TP and its eastern margin. Monophyly of *Chaparana* is not



**Figure 2** Bayesian inference (A) and Maximum-likelihood (B) tree inferred from the concatenated mtDNA and nuDNA sequence alignment. Numbers at branch nodes refer to posterior probabilities  $\geq 0.9$  and bootstrap values  $> 70$ , respectively. For IQ-TREE topology see supplemental Fig. S2.

Full-size [DOI: 10.7717/peerj.11793/fig-2](https://doi.org/10.7717/peerj.11793/fig-2)

supported in the analyses if secondary structure of 16S is ignored. All *Paa* species together form the most species diverse clade.

Since the placement of *Allopaa* is of particular interest in terms of the origin and past biogeography of Pains, we tested the resulting topologies of major clades: BI tree considering secondary structure information of 16S,  $t_1$ : (*Allopaa* (*Nanorana*)); RAXML/BI without secondary structure information,  $t_2$ : ((*Chaparana*, *Allopaa*)(*Nanorana* sensu stricto, *Paa* sensu stricto)).

The AU test does not reject one of the two placement models for *Allopaa* (Table 1), as do the results of all other IQ-TREE tests. However, the BF of 28 (ss) and 32 (ps), based on the model likelihood values estimated with BEAST, strongly rejects a basal placement of *Allopaa* relative to the genus *Nanorana* in favor of the topology seen in the ML tree. Similarly, the marginal likelihoods calculated based on the runs considering the secondary structure of 16S were significantly higher for the unconstrained model (Table 1). Thus, the phylogenetic position of *Allopaa* as sister clade to *Chaparana* seems to be most likely, thereby making the *Nanorana* genus paraphyletic.

### Divergence times in spiny frogs

Dating analysis suggests an origin of Pains (*Allopaa*, *Chrysopaa*, *Nanorana*, *Quasipaa*) in the mid Oligocene (28.21 Ma, 20.11–35.18 Ma), what is in the range of previous



**Table 1** Tree topology comparisons between the two models of *Allopa* placements. Models ( $t_1$ ,  $t_2$ ) are compared based on Bayesian factor (BF) using BEAST, as well as the unbiased (AU) test (Shimodaira 2002), bootstrap proportion using RELL method (Kishino, Miyata & Hasegawa, 1990), Kishino-Hasegawa (KH) test (Kishino & Hasegawa, 1989), Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa, 1999), and expected likelihood weights (ELW) using IQ-TREE; BF was also calculated for a hard constraint on *Chaparana* and *Allopa* (A+Ch) vs. an unconstraint constellation using the stepping-stone approach in MrBayes and considering the secondary structure information of 16S.

Topology	ps	ss	2lnBF	logL	deltaL	bp-RELL	p-KH	p-SH	c-ELW	p-AU
$t_1$ (A(N))	-15471	-15477	ps: 32	-14164.109	2.458	0.397+	0.383+	0.383+	0.399+	0.383+
$t_2$ ((A+Ch)(P,N))	<b>-15455</b>	<b>-15463</b>	ss: 28	<b>-14161.652</b>	0	0.603+	0.617+	1+	0.601+	0.617+
(A+Ch)		<b>-16472</b>								
unconstraint		-16500	ss: 56							

**Notes.**

A, *Allopa*; C, *Chaparana*; N, *Nanorana* (genus); P, *Paa*; ps, path sampling log marginal likelihood; ss, stepping-stone log marginal likelihood; +, a tree is not rejected if its  $p$ -value > 0.05. Bold log marginal likelihood values indicate the model most favored by a method (higher is better).

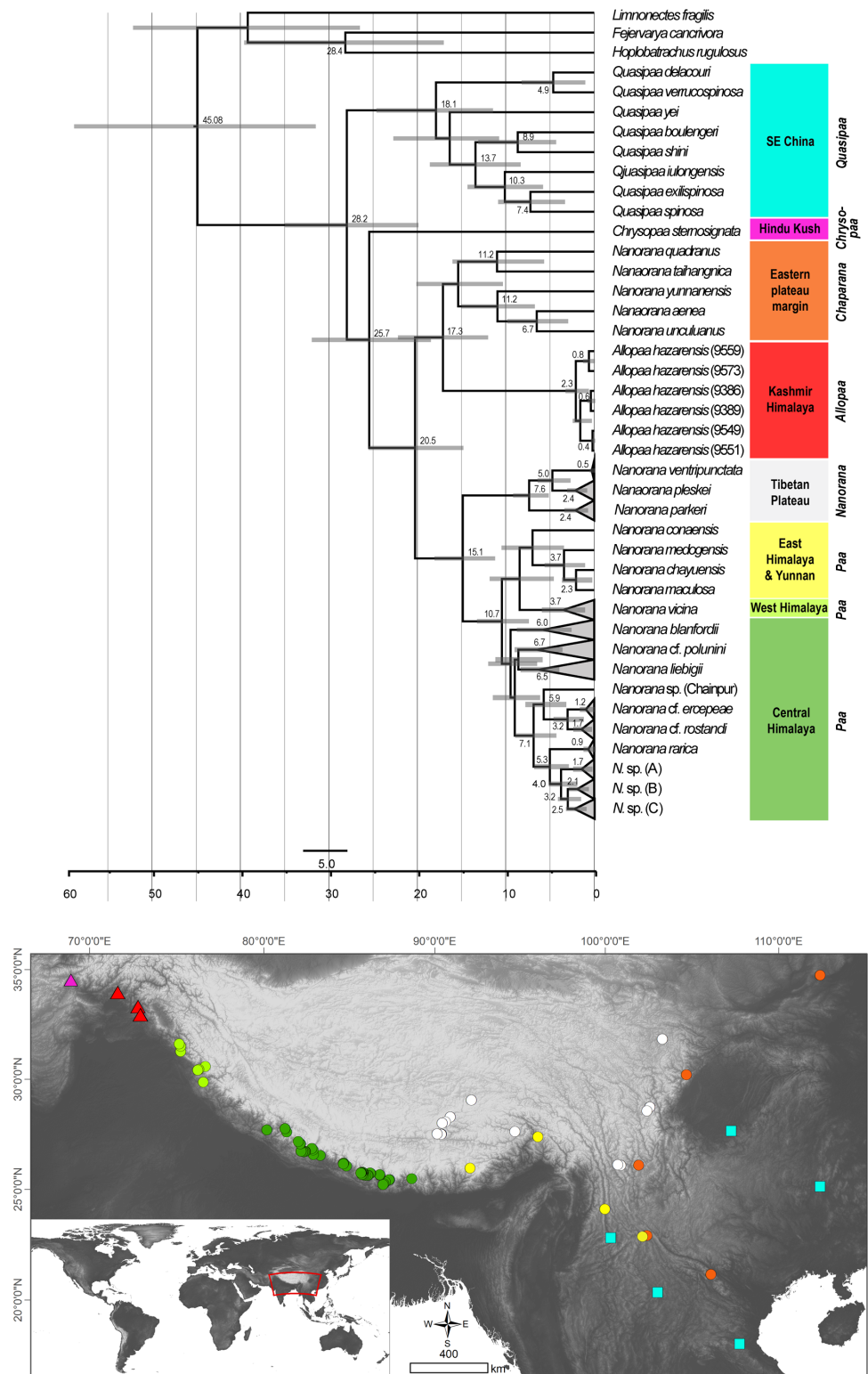
estimations ([Che et al., 2010](#); [Hofmann et al., 2019](#); [Sun et al., 2018](#)) ([Fig. 3](#)). The age of Himalayan-Tibetan spiny frogs (*Allopaa*, *Chrysopaa*, *Nanorana*) is estimated to be 25.7 Ma (18.70–32.16). Within crown *Allopaa*+*Nanorana*, the clade comprising the montane *Chaparana* and West-Himalayan *Allopaa* split from the Central/East Himalayan and Tibetan *Nanorana* (subgenera *Paa* and *Nanorana*) in the early Miocene, around 20 Ma, followed by the separation of *Chaparana* and *Allopaa* ca. 3 million years later. The divergence of the nominal *Nanorana* (endemic to the TP) from *Paa* (Greater Himalaya) occurred around 15 Ma (11.45–18.27 Ma). This estimate is close to the age of 13 Ma (7–25 Ma) calculated by [Sun et al. \(2018\)](#), and 10–12 Ma estimated by [Wiens et al. \(2009\)](#).

Diversification of Central Himalayan *Paa* clades has taken place continuously during the whole Mid to Late Miocene. Most of the main lineages within *Paa* were present at least in the late Miocene, and nearly all species are not younger than the Pliocene.

## DISCUSSION

We here report the first phylogeny of the westernmost HTO Paini taxa *Chrysopaa sternosignata* and *Allopaa hazarensis* in the context of their closest relatives. Our work based on sequence information of *A. hazarensis* specimens from the foothills of the Kashmir Himalaya, a previously published data set ([Hofmann et al., 2019](#)), and additional sequence data of *C. sternosignata* from the Hindu Kush Mts. in Afghanistan available from GenBank. The study provides evidence for an early-Miocene evolution of Himalayan Paini, which is ultimately linked to the paleoecological evolution of the HTO.

Consistent with our previous results ([Hofmann et al., 2019](#)), the Southeast Asian genus *Quasipaa* is sister to all other spiny frogs. Most remarkable, the monotypic *Chrysopaa* is placed basally relative to *Nanorana* and *Allopaa*, supporting the presence of ancestral Paini lineages in the far northwestern part of the HTO, which is diametrically opposite end of the HTO with respect to the ancestral area of spiny frogs that is assumed to be the Paleogene East or Southeast China ([Che et al., 2010](#); [Hofmann et al., 2019](#)). Thus, it can be assumed that the ancestor of *Chrysopaa* appeared elsewhere near the eastern margin of the HTO during the late Oligocene-early Miocene. If so, it implies that members of the *Chrysopaa* stem group must have been temporarily present in the interior of the HTO during the following time, to enable a range expansion up to the western margin of the mountains system. Given this scenario, the climatic preferences of ancestral spiny frogs are of particular interest. Amphibians are particularly sensitive to changes in hydric and thermal environmental conditions ([Kerby et al., 2010](#); [Mitchell & Janzen, 2010](#); [Ochoa-Ochoa et al., 2012](#); [Stuart et al., 2004](#)), and many of them show remarkable evolutionary stasis in ecological niches, suggesting that dispersal might have been historically constrained between similar climatic conditions ([Konzak & Wiens, 2010](#); [Wiens, 2011](#), and references therein). Since all species of the most basal clade *Quasipaa* occur under subtropical climate ([Frost, 2021](#); [Bain & Hurley, 2011](#)), a similar temperature preference might be assumed for the *Chrysopaa* ancestor. We suspect that this preference has not changed significantly during the Neogene period as *C. sternosignata* occurs under subtropical to warm temperate climate conditions in the colline zone of the Hindu Kush Mts. and the Kashmir valley



**Figure 3** Ultrametric time-calibrated phylogeny generated with BEAST2 based on the concatenated sequence data of spiny frogs. Grey bars specify the 95% HPD for the respective nodes; ages are shown for nodes that are supported by Bayesian posterior probability  $\geq 0.95$ . Colors at clades correspond to the lower distribution map.

Full-size DOI: 10.7717/peerj.11793/fig-3

(Khan, 2006; Sarwar et al., 2016; Wagner et al., 2016). Consequently, a subtropical climate associated with sufficient humidity suitable for amphibians might have existed in large parts of the late Oligocene-Tibet to allow a trans-Tibetan dispersal of *Chrysopaa* stem group members. Interestingly, basal divergences of West Himalayan taxa are also known from the gekkonid genus *Cyrtodactylus*, dating even back to the early Eocene and demonstrating that ancestral *Cyrtodactylus* were present in the “proto-Himalayan region” (Argawal et al., 2014). The topology of the genus provides striking parallels to the Pains tree and indications in support of a Tibetan-origin (Hofmann et al., 2017; Schmidt et al., 2012; Hofmann et al., 2019) of *Cyrtodactylus* followed by a trans-Tibetan dispersal of ancestral lineages to the northwestern HTO margin.

Also unexpected are our results with respect to the phylogenetic position and timing of the evolution of *Allopaa* from the foothills of the Kashmir Himalaya. This group evolved during the early to mid-Miocene most parsimoniously as sister clade to *Chaparana*, although a basal position of *Allopaa* relative to the genus *Nanorana* cannot be entirely excluded. Species of *Chaparana* occur along the eastern margin of the HTO and therewith at the opposite end of the HTO where *Allopaa* is distributed. A similar paradoxical pattern can be found in the above mentioned *Cyrtodactylus* group (Argawal et al., 2014) and in Broscini ground beetles (Schmidt, Wrase & Sciaky, 2013) with species from the western Himalaya being most closely related to those from the Eastern Himalaya and Southeast Asia. *Chaparana* and *Allopaa* together constitute most likely the sister clade to the Tibetan *Nanorana* and Himalayan *Paa*, which indicates that *Nanorana* might be paraphyletic with respect to *Allopaa*. However, to prevent instability in taxonomic nomenclature, at this stage we refrain from proposing any taxonomic changes until further evidence is available. Our results also show that *Allopaa* is phylogenetically not related to the biogeographically neighboring Himalayan spiny frogs. This finding is crucial with respect to the ancestral distributional area of the *Chaparana*+*Allopaa* clade and their ancestral habitat preferences. Recent species of *Chaparana* occur in the colline and lower montane zone along the eastern margin of the HTO and the easterly neighbored mountains and, thus, immediately adjacent to (or overlapping with) the supposed ancestral area of spiny frogs (Che et al., 2010; Hofmann et al., 2019). Similar as assumed for *Chrysopaa*, the ancestor of *Allopaa* must have been dispersed across a moderately elevated Tibetan Plateau, although about eight million years later than the ancestor of *Chrysopaa*. Since species of *Allopaa* occur under warm-temperate conditions in the colline to lower montane zone (comparable to those of its sister group *Chaparana*; Ahmed et al., 2020), similar temperature preferences can be assumed for ancestral *Allopaa*. Therefore, the supposed trans-Tibet dispersal event of this lineage implies the presence of warm temperate conditions in significant parts of Tibet’s interior at least up to the early-mid Miocene boundary. This is supported by the evidence of subtropical to warm-temperate fossil floras in the Qiabulin basin at 21–19 Ma (Ding et al., 2014), Lunpola basin at 25.5–19.8 Ma (Sun et al., 2014) and in the Kailas basin at about 23.3 Ma (Ai et al., 2019). Due to the progressive uplift of Tibet and the associated continuous cooling of the regional climates, the *Allopaa* stem group members might have successively been lost to extinction. Today’s absence of members of *Chaparana* and *Allopaa* in the high montane zone throughout the HTO suggests that these lineages were probably

not able to adapt fast enough to the conditions that resulted from the dramatically changing environment. Alternatively, a westward and northwestward spread of ancestral *Allopa* using subtropical to warm-temperate habitats which paralleling the southern slopes of the Himalaya must also be considered. However, this model is very unlikely, as it would imply extinction of all ancestral lineages in vast areas covering almost the whole Himalayan mountain arc. Considering that since the onset of surface uplift subtropical to warm temperate environments were continuously present along the Himalayan southern face (Hongfu, 1994; Sanyal & Sinha, 2010; Xu et al., 2012), such radical extinction or turnover is implausible given the recent and former ecological conditions in this area. Moreover, the absence of *Allopa*, but occurrence of many spiny frogs of the subgenus *Paa* along the southern slopes of the eastern, central, and western Himalaya north to the Indian Himachal Pradesh, contradicts this extinction scenario.

Unlike spiny frogs of the taxa *Chrysopaa*, *Allopa* and *Chaparana* which are restricted to the subtropical to warm temperate climate, many representatives of the *Nanorana* + *Paa* clade are adapted to colder habitats and occur in the high montane, subalpine, and alpine zones of the HTO. The evolutionary late appearance of this clade is indicative for the minimum age of high-altitude environments in the HTO: Although spiny frogs were present in the area since at least the Paleogene/Neogene boundary, cold-adapted species did not evolve before ca. 15 Ma (Fig. 3). This is a strong hint that extensive high-altitude environments were present in the HTO from mid-Miocene at earliest.

## CONCLUSIONS

We provide the first phylogenetic study of spiny frogs that comprises the two westernmost Himalayan taxa *Allopa* and *Chrysopaa*. Our findings suggest a late Oligocene to early Miocene dispersal of two subtropical respective warm temperate lineages, *Chrysopaa* and *Allopa*, from the ancestral area of spiny frogs in SE Asia across the HTO into its far northwestern part. This dispersal scenario is crucial with respect to the long-standing debate regarding the paleoenvironmental and paleoelevational development of the TP. Given the stem age of subtropical *Chrysopaa* of ca. 26 Mya and the divergence time of 17 Mya between warm temperate *Allopa* and *Chaparana*, our results strongly indicate the large-scale presence of subtropical environments north of today's Greater Himalaya until the late Oligocene, and of warm temperate climates until the late Miocene. This contrasts with geoscientific models of the paleoelevational evolution of the TP which assume large scale surface uplift close to present heights until the mid-Oligocene (e.g., Kapp et al., 2007; Mulch & Chamberlain, 2006; Tapponnier et al., 2001; Wang et al., 2008; Wang et al., 2014), and which are widely used in recent biogeographic studies to develop evolutionary scenarios in different species groups (e.g., Favre et al., 2015; Favre et al., 2016; Renner, 2016; Mosbrugger et al., 2018). However, over the last decade a growing number of fossil data provide evidence for the presence of tropical to warm temperate floras and freshwater fishes in central Tibet during the late Paleogene until the early Neogene (Song et al., 2010; Su et al., 2019; Wei et al., 2016; Wu et al., 2017). Consistent with these findings our results support the recent concept proposed by Spicer and colleagues (Spicer et al., 2020), which

assumes that the TP was not uplifted as a whole, but instead, a deep wide east–west oriented valley occurred in the Tibetan interior before the final plateau formation. We suspect that this supposed valley represents the migration corridor of the ancestral *Chrysopaa* and *Allopaa* lineages, which today are represented by the two relict taxa, *C. sternosignata* and *A. hazarensis*, endemic to the region of the Hindu Kush and Kashmir Himalaya. This scenario is in line with and adds to the Tibetan-origin hypothesis of the paleo-Tibetan fauna (Hofmann et al., 2017; Hofmann et al., 2019; Schmidt, Wrase & Sciaky, 2013). Disjunct distribution patterns of species groups between the eastern and western part of the HTO, as we demonstrate here for spiny frogs, have been also observed in *Cyrtodactylus* (Argawal et al., 2014, see Discussion), and in Broscini ground beetles, with the genus *Eobroscus* widely distributed in East Asia and Indochina and with *Kashmirobrosus* endemic to a small part of the Kashmir Himalaya (Schmidt, Wrase & Sciaky, 2013). Moreover, the Kashmir Himalaya is well-known for the occurrence of several highly endemic ground beetles (Schmidt et al., 2012). We expect that numerous additional lineages endemic to the Kashmir Himalaya will be identified in future which may contribute to resolve the evolution of the HTO. We therefore encourage further and systematic research in this area and the use of more powerful molecular data, for example, through the use of genomic sequencing to better understand the evolution and Cenozoic history of Himalayan biodiversity against the background of existing geological scenarios.

## ACKNOWLEDGEMENTS

We thank Sandra Kukowa, Anja Bodenheim, and Jana Poláková for technical support in the lab. We are also grateful to students and colleagues of RM in Pakistan for their assistance during the field work.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

This work was funded by the German Research Foundation (DFG, grant no. HO 3792/8-1 to Sylvia Hofmann), and by the Slovak Research and Development Agency (grant no. APVV-19-0076 to Daniel Jablonski). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:

The German Research Foundation: HO 3792/8-1.

The Slovak Research and Development Agency: APVV-19-0076.

### Competing Interests

The authors declare there are no competing interests.

### Author Contributions

- Sylvia Hofmann conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Daniel Jablonski conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, contributed equally to this work as senior author, and approved the final draft.
- Spartak N. Litvinchuk and Razaqat Masroor performed the experiments, prepared figures and/or tables, and approved the final draft.
- Joachim Schmidt performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, contributed equally to this work as senior author, and approved the final draft.

### Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Pakistan Museum of Natural History, Islamabad, Pakistan approved this study (PMNH/EST-1(89)/05).

### DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The data are available at GenBank ([Table S1](#)).

### Data Availability

The following information was supplied regarding data availability:

The data are available at GenBank ([Table S1](#)).

The analysis pipelines used in this study are publicly accessible:

<https://www.megasoftware.net/>;

<https://cme.h-its.org/exelixis/web/software/raxml/>;

<http://www.iqtree.org/>;

<https://nbisweden.github.io/MrBayes/download.html>;

<https://www.robertlanfear.com/partitionfinder/>;

<https://beast.community/>;

<https://www.beast2.org/>.

### Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.11793#supplemental-information>.

## REFERENCES

- Ahmed W, Rais M, Saeed M, Gill S, Akram S. 2020. Site occupancy of two endemic stream frogs in different forest types in Pakistan. *Herpetological Conservation and Biology* 15:506–511.

- Ai K, Shi G, Zhang K, Ji J, Song B, Shen T, Guo S. 2019.** The uppermost Oligocene Kailas flora from southern Tibetan Plateau and its implications for the uplift history of the southern Lhasa terrane. *Palaeogeography, Palaeoclimatology, Palaeoecology* **515**:143–151 DOI [10.1016/j.palaeo.2018.04.017](https://doi.org/10.1016/j.palaeo.2018.04.017).
- Antonelli A, Nylander JA, Persson C, Sanmartin I. 2009.** Tracing the impact of the Andean uplift on Neotropical plant evolution. *Proceedings of the National Academy of Sciences of the United States of America* **106**:9749–9754 DOI [10.1073/pnas.0811421106](https://doi.org/10.1073/pnas.0811421106).
- Argawal I, Bauer AM, Jackman TR, Karanth KP. 2014.** Insights into Himalayan biogeography from geckos: a molecular phylogeny of *Cyrtodactylus* (Squamata: Gekkonidae). *Molecular Phylogenetics and Evolution* **80**:145–155 DOI [10.1016/j.ympev.2014.07.018](https://doi.org/10.1016/j.ympev.2014.07.018).
- Avise JC. 2009.** Phylogeography: retrospect and prospect. *Journal of Biogeography* **36**:3–15 DOI [10.1111/j.1365-2699.2008.02032.x](https://doi.org/10.1111/j.1365-2699.2008.02032.x).
- Avise JC, Nelson WS, Bowen BW, Walker D. 2000.** Phylogeography of colonially nesting seabirds, with special reference to global matrilineal patterns in the sooty tern (*Sterna fuscata*). *Molecular Ecology* **9**:1783–1792 DOI [10.1046/j.1365-294x.2000.01068.x](https://doi.org/10.1046/j.1365-294x.2000.01068.x).
- Bain RH, Hurley MM. 2011.** A biogeographic synthesis of the amphibians and reptiles of Indochina. *Bulletin of the American Museum of Natural History* **360**:1–138 DOI [10.1206/360.1](https://doi.org/10.1206/360.1).
- Bouckaert RR, Drummond AJ. 2017.** bModelTest: bayesian phylogenetic site model averaging and model comparison. *BMC Evolutionary Biology* **17**:1–11 DOI [10.1186/s12862-016-0855-1](https://doi.org/10.1186/s12862-016-0855-1).
- Bouckaert R, Heled J, Kuhnert D, Vaughan T, Wu CH, Xie D, Suchard MA, Rambaut A, Drummond AJ. 2014.** BEAST 2: a software platform for Bayesian evolutionary analysis. *PLOS Computational Biology* **10**:e1003537 DOI [10.1371/journal.pcbi.1003537](https://doi.org/10.1371/journal.pcbi.1003537).
- Boulenger GA. 1920.** A monograph of the South Asian, Papuan, Melanesian and Australian frogs of the genus *Rana*. *Records of the Indian Museum* **20**:1–226.
- Che J, Hu JS, Zhou WW, Murphy RW, Papenfuss TJ, Chen MY, Rao DQ, Li PP, Zhang YP. 2009.** Phylogeny of the Asian spiny frog tribe Paini (Family Dicroglossidae) sensu Dubois. *Molecular Phylogenetics and Evolution* **50**:59–73 DOI [10.1016/j.ympev.2008.10.007](https://doi.org/10.1016/j.ympev.2008.10.007).
- Che J, Zhou WW, Hu JS, Yan F, Papenfuss TJ, Wake DB, Zhang YP. 2010.** Spiny frogs (Paini) illuminate the history of the Himalayan region and Southeast Asia. *Proceedings of the National Academy of Sciences of the United States of America* **107**:13765–13770 DOI [10.1073/pnas.1008415107](https://doi.org/10.1073/pnas.1008415107).
- Chernomor O, von Haeseler A, Minh BQ. 2016.** Terrace aware data structure for phylogenomic inference from supermatrices. *Systematic Biology* **65**:997–1008 DOI [10.1093/sysbio/syw037](https://doi.org/10.1093/sysbio/syw037).
- Ding WN, Ree RH, Spicer RA, Xing YW. 2020.** Ancient orogenic and monsoon-driven assembly of the world's richest temperate alpine flora. *Science* **369**:578–581 DOI [10.1126/science.abb4484](https://doi.org/10.1126/science.abb4484).



- Ding L, Xu Q, Yue Y, Wang HJ, Cai F, Li S. 2014.** The Andean-type Gangdese Mountains: paleoelevation record from the Paleocene–Eocene Linzhou Basin. *Earth and Planetary Science Letters* **392**:250–264 DOI [10.1016/j.epsl.2014.01.045](https://doi.org/10.1016/j.epsl.2014.01.045).
- Donoghue PCJ, Benton MJ. 2007.** Rocks and clocks: calibrating the Tree of Life using fossils and molecules. *Trends in Ecology and Evolution* **22**:424–431 DOI [10.1016/j.tree.2007.05.005](https://doi.org/10.1016/j.tree.2007.05.005).
- Drummond AJ, Rambaut A. 2007.** BEAST: bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**:214 DOI [10.1186/1471-2148-7-214](https://doi.org/10.1186/1471-2148-7-214).
- Dubois A. 1975.** A new sub-genus (*Paa*) and three new species of the genus *Rana*, Remarks on the phylogeny of Ranidae (Amphibia, Anura) (translated from French). *Bulletin Du Muséum National D'Histoire Naturelle Zoologie* **231**:1093–1115.
- Edgar RC. 2004.** MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**:1792–1797 DOI [10.1093/nar/gkh340](https://doi.org/10.1093/nar/gkh340).
- Favre A, Michalak I, Chen CH, Wang JC, Pringle JS, Matuszak S, Sun H, Yuan YM, Struwe L, Muellner-Riehl AN. 2016.** Out-of-Tibet: the spatio-temporal evolution of *Gentiana* (Gentianaceae). *Journal of Biogeography* **43**:1967–1978 DOI [10.1111/jbi.12840](https://doi.org/10.1111/jbi.12840).
- Favre A, Paeckert M, Pauls SU, Jaehnig SC, Uhl D, Michalak I, Muellner-Riehl AN. 2015.** The role of the uplift of the Qinghai-Tibetan Plateau for the evolution of Tibetan biotas. *Biological Reviews* **90**:236–253.
- Frost DR. 2021.** Amphibian species of the world: an online reference. Version 6.0. Electronic Database. <http://research.amnh.org/herpetology/amphibia/index.html> (accessed on May 2021).
- Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010.** New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**:307–321 DOI [10.1093/sysbio/syq010](https://doi.org/10.1093/sysbio/syq010).
- Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. 2018.** UFBoot2: improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution* **35**:518–522 DOI [10.1093/molbev/msx281](https://doi.org/10.1093/molbev/msx281).
- Hofmann S, Baniya CB, Litvinchuk SN, Miede G, Li JT, Schmidt J. 2019.** Phylogeny of spiny frogs *Nanorana* (Anura: Dicroglossidae) supports a Tibetan origin of a Himalayan species group. *Ecology and Evolution* **9**:14498–14511 DOI [10.1002/ece3.5909](https://doi.org/10.1002/ece3.5909).
- Hofmann S, Stoeck M, Zheng Y, Ficetola FG, Li JT, Scheidt U, Schmidt J. 2017.** Molecular phylogenies indicate a Paleo-Tibetan origin of himalayan lazy toads (*Scutigera*). *Scientific Reports* **7**:3308 DOI [10.1038/s41598-017-03395-4](https://doi.org/10.1038/s41598-017-03395-4).
- Hongfu Y. 1994.** Implications of palaeobiogeographical provincialization for the Mesozoic and Cenozoic palaeoclimate and palaeodrainage of China. In: Hongfu Y, ed. *The palaeobiogeography of China*. Oxford Biogeography Series No. 8, New York: Oxford University Press, 333–344.
- Hoorn C, Mosbrugger V, Mulch A, Antonelli A. 2013.** Biodiversity from mountain building. *Nature Geoscience* **6**:154 DOI [10.1038/ngeo1742](https://doi.org/10.1038/ngeo1742).

- Huang Y, Hu JS, Wang B, Song Z, Zhou C-J, Jiang J. 2016. Integrative taxonomy helps to reveal the mask of the genus *Gynandropaa* (Amphibia: Anura: Dicroglossidae). *Integrative Zoology* 11:134–150 DOI 10.1111/1749-4877.12169.
- Jiang JP, Dubois A, Ohler A, Tillier A, Chen XH, Xie F, Stock M. 2005. Phylogenetic relationships of the tribe Paini (Amphibia, Anura, Ranidae) based on partial sequences of mitochondrial 12s and 16s rRNA genes. *Zoological Science* 22:353–362 DOI 10.2108/zsj.22.353.
- Johansson US, Alstrom P, Olsson U, Ericson PGR, Sundberg P, Price TD. 2007. Build-up of the Himalayan avifauna through immigration: a biogeographical analysis of the *Phylloscopus* and *Seicercus* warblers. *Evolution* 61:324–333 DOI 10.1111/j.1558-5646.2007.00024.x.
- Kapp P, De Celles PG, Leier AL, Fabijanic JM, He S, Pullen A, Gehrels GE. 2007. The Gangdese retroarc thrust belt revealed. *GSA Today* 17:4–9.
- Kass RE, Raftery AE. 1995. Bayes factors. *Journal of the American Statistical Association* 90:773–795 DOI 10.1080/01621459.1995.10476572.
- Kerby JL, Richards-Hrdlicka KL, Storfer A, Skelly DK. 2010. An examination of amphibian sensitivity to environmental contaminants: are amphibians poor canaries? *Ecological Letters* 13:60–67 DOI 10.1111/j.1461-0248.2009.01399.x.
- Khan MS. 2006. *Amphibians and reptiles of Pakistan*. Malabar, Florida: Krieger Publishing Company, 311 pp.
- Kishino H, Hasegawa M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. *Journal of Molecular Evolution* 29:170–179 DOI 10.1007/BF02100115.
- Kishino H, Miyata T, Hasegawa M. 1990. Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. *Journal of Molecular Evolution* 31:151–160 DOI 10.1007/BF02109483.
- Konzak KH, Wiens JJ. 2010. Niche conservatism drives elevational diversity patterns in Appalachian salamanders. *The American Naturalist* 176:40–54 DOI 10.1086/653031.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35:1547–1549 DOI 10.1093/molbev/msy096.
- Kutzbach JE, Guetter PJ, Ruddiman WF, Prell WL. 1989. The sensitivity of climate to late Cenozoic uplift in southern Asia and the American west: numerical experiments. *Journal Geophysical Research* 94:18393–18407 DOI 10.1029/JD094iD15p18393.
- Lanfear R, Calcott B, Ho SY, Guindon S. 2012. Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29:1695–1701 DOI 10.1093/molbev/mss020.
- Lartillot N, Philippe H. 2006. Computing Bayes Factors using thermodynamic integration. *Systematic Biology* 55:195–207 DOI 10.1080/10635150500433722.
- Luebert F, Muller LA. 2015. Effects of mountain formation and uplift on biological diversity. *Frontiers in Genetics* 6:54.
- Miao Y, Wu F, Chang H, Fang XM, Deng T, Sun J, Jin C. 2016. A Late-Eocene palynological record from the Hoh Xil Basin, northern Tibetan Plateau, and its

- implications for stratigraphic age, paleoclimate and paleoelevation. *Gondwana Research* **31**:241–252 DOI [10.1016/j.gr.2015.01.007](https://doi.org/10.1016/j.gr.2015.01.007).
- Miller MA, Pfeiffer W, Schwartz T. 2010.** Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Proceedings of the Gateway Computing Environments Workshop (GCE)*. New Orleans, LA, 1–8.
- Mitchell NJ, Janzen FJ. 2010.** Temperature-dependent sex determination and contemporary climate change. *Sex Development* **4**:129–140 DOI [10.1159/000282494](https://doi.org/10.1159/000282494).
- Molnar P, Boos WR, Battasti DS. 2010.** Orographic controls on climate and paleoclimate of Asia: thermal and mechanical roles for the Tibetan Plateau. *Annual Review of Earth and Planetary Sciences* **38**:77–102 DOI [10.1146/annurev-earth-040809-152456](https://doi.org/10.1146/annurev-earth-040809-152456).
- Molnar P, England P, Martiod J. 1993.** Mantle dynamics, uplift of the Tibetan Plateau and the Indian monsoon development. *Reviews of Geophysics* **34**:357–396.
- Mosbrugger V, Favre A, Muellner-Riehl AN, Päckert M, Mulch A. 2018.** Cenozoic evolution of geo-biodiversity in the Tibeto-Himalayan region. In: Hoorn C, Perrigo A, Antonelli A, eds. *Mountains, climate and biodiversity*. Oxford, UK: Wiley & Sons, 429–448.
- Mulch A, Chamberlain CP. 2006.** Earth science—The rise and growth of Tibet. *Nature* **439**:670–671 DOI [10.1038/439670a](https://doi.org/10.1038/439670a).
- Mulch A, Chamberlain CP. 2018.** Stable Isotope Peleoaltimetry: paleotopography as a key element in the evolution of landscape and life. In: Hoorn C, Perrigo A, Antonelli A, eds. *Mountains, climate and biodiversity*. Oxford, UK: Wiley & Sons, 81–94.
- Murphy MA, Yin A, Harrison TM, Durr SB, Chen Z, Ryerson FJ, Kidd WSF, Wang X, Zhou X. 1997.** Did the Indo-Asian collision alone create the Tibetan plateau? *Geology* **25**:719–722 DOI [10.1130/0091-7613\(1997\)025<0719:DTIACA>2.3.CO;2](https://doi.org/10.1130/0091-7613(1997)025<0719:DTIACA>2.3.CO;2).
- Ochoa-Ochoa LM, Rodríguez P, Mora F, Flores-Villela O, Whittaker RJ. 2012.** Climate change and amphibian diversity patterns in Mexico. *Biological Conservation* **150**:94–102 DOI [10.1016/j.biocon.2012.03.010](https://doi.org/10.1016/j.biocon.2012.03.010).
- Ohler A, Dubois A. 2006.** Phylogenetic relationships and generic taxonomy of the tribe Paini (Amphibia, Anura, Ranidae, Dicroglossinae), with diagnoses of two new genera. *Zoosystema* **28**:769–784.
- Pyron RA, Wiens JJ. 2011.** A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution* **61**:543–583 DOI [10.1016/j.ympev.2011.06.012](https://doi.org/10.1016/j.ympev.2011.06.012).
- Quade J, Brecker DO, Daëron M, Eiler J. 2011.** The Paleoaltimetry of Tibet: an isotopic perspective. *American Journal of Science* **311**:77–115 DOI [10.2475/02.2011.01](https://doi.org/10.2475/02.2011.01).
- Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018.** Posterior summarization in bayesian phylogenetics using Tracer 1.7. *Systematic Biology* **67**:901–904 DOI [10.1093/sysbio/syy032](https://doi.org/10.1093/sysbio/syy032).
- Raymo ME, Ruddiman WF. 1992.** Tectonic forcing of late Cenozoic climate. *Nature* **359**:117–122 DOI [10.1038/359117a0](https://doi.org/10.1038/359117a0).
- Renner SS. 2016.** Available data point to a 4-km-high Tibetan Plateau by 40Ma, but 100 molecular-clock papers have linked supposed recent uplift to young node ages. *Journal of Biogeography* **43**:1479–1487 DOI [10.1111/jbi.12755](https://doi.org/10.1111/jbi.12755).

- Richardson JE, Madriñán S, Gómez-Gutiérrez MC, Valderrama E, Luna J, Banda RK, Serrano J, Torres M, Jara O, Aldana A, Cortés BR, Sánchez D, Montes C. 2018.** Using dated molecular phylogenies to help reconstruct geological, climatic, and biological history: examples from Colombia. *Geological Journal* **2018**:1–9 DOI [10.1155/2018/3749725](https://doi.org/10.1155/2018/3749725).
- Ronquist F, Teslenko M, Van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012.** MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**:539–542 DOI [10.1093/sysbio/sys029](https://doi.org/10.1093/sysbio/sys029).
- Rowley DB, Currie BS. 2006.** Palaeo-altimetry of the late Eocene to Miocene Lunpola basin, Central Tibet. *Nature* **439**:677–681 DOI [10.1038/nature04506](https://doi.org/10.1038/nature04506).
- Sanyal P, Sinha R. 2010.** Evolution of the Indian summer monsoon: synthesis of continental records. In: Clift PD, Tada R, Zheng H. eds. Monsoon evolution and tectonics–Climate linkage in Asia. *Geological Society London, Special Publications* **342**:153–183 DOI [10.1144/SP342.11](https://doi.org/10.1144/SP342.11).
- Sarwar MK, Malik MF, Hussain M, Azam I, Iqbal W, Ashiq U. 2016.** Distribution and current status of amphibian fauna of Pakistan: a review. *Electronic Journal of Biology* **12**:243–246.
- Schmidt J, Opgenoorth L, Holl S, Bastrop R. 2012.** Into the Himalayan exile: the phylogeography of the ground beetle *Ethira* clade supports the Tibetan origin of forest-dwelling Himalayan species groups. *PLOS ONE* **7**:e45482 DOI [10.1371/journal.pone.0045482](https://doi.org/10.1371/journal.pone.0045482).
- Schmidt J, Wrase DW, Sciaky R. 2013.** Description of *Kashmirobrosicus* gen. n. with two new species from the Northwest Himalaya, and remarks on the East Asian genus *Eobrosicus* Kryzhanovskij, 1951 (Coleoptera: Carabidae: Broscini). *Journal of Natural History* **47**:2671v2689.
- Schoniger M, von Haeseler A. 1999.** Toward assigning helical regions in alignments of ribosomal RNA and testing the appropriateness of evolutionary models. *Journal of Molecular Evolution* **49**:691–698 DOI [10.1007/PL00006590](https://doi.org/10.1007/PL00006590).
- Shimodaira H. 2002.** An approximately unbiased test of phylogenetic tree selection. *Systematic Biology* **51**:492–508 DOI [10.1080/10635150290069913](https://doi.org/10.1080/10635150290069913).
- Shimodaira H, Hasegawa M. 1999.** Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* **16**:1114–1116 DOI [10.1093/oxfordjournals.molbev.a026201](https://doi.org/10.1093/oxfordjournals.molbev.a026201).
- Song XY, Spicer RA, Yang J, Yao YF, Li CS. 2010.** Pollen evidence for an Eocene to Miocene elevation of central southern Tibet predating the rise of the High Himalaya. *Palaeogeography, Palaeoclimatology, Palaeoecology* **297**:159–168 DOI [10.1016/j.palaeo.2010.07.025](https://doi.org/10.1016/j.palaeo.2010.07.025).
- Spicer RA. 2017.** Tibet, the Himalaya, Asian monsoons and biodiversity—In what ways are they related? *Plant Diversity* **39**:233–244 DOI [10.1016/j.pld.2017.09.001](https://doi.org/10.1016/j.pld.2017.09.001).
- Spicer RA, Su T, Valdes PJ, Farnsworth A, Wu F-X, Shi G, Ste V, Zhou Z. 2020.** Why ‘the uplift of the Tibetan Plateau’ is a myth? *National Science Review* **8**:nwaa091.

- Stamatakis A. 2014.** RAxML Version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**:1312–1313 DOI [10.1093/bioinformatics/btu033](https://doi.org/10.1093/bioinformatics/btu033).
- Steinbauer MJ, Field R, Grytnes JA, Trigas P, Ah-Peng C, Attorre F, Birks HJB, Borges PAV, Cardoso P, Chou CH, De Sanctis M, De Sequeira MM, Duarte MC, Elias RB, Fernandez-Palacios JM, Gabriel R, Gereau RE, Gillespie RG, Greimler J, Harter DEV, Huang TJ, Irl SDH, Jeanmonod D, Jentsch A, Jump AS, Kueffer C, Nogue S, Otto R, Price J, Romeiras MM, Strasberg D, Stuessy T, Svenning JC, Vetaas OR, Beierkuhnlein C. 2016.** Topography-driven isolation, speciation and a global increase of endemism with elevation. *Global Ecology and Biogeography* **25**:1097–1107 DOI [10.1111/geb.12469](https://doi.org/10.1111/geb.12469).
- Stocsits RR, Letsch H, Hertel J, Misof B, Stadler PF. 2009.** Accurate and efficient reconstruction of deep phylogenies from structured RNAs. *Nucleic Acids Research* **37**:6184–6193 DOI [10.1093/nar/gkp600](https://doi.org/10.1093/nar/gkp600).
- Stoliczka F. 1872.** Notes on some new species of Reptilia and Amphibia, collected by Dr. W. Waagen in North-western Punjab. *Proceedings of the Asiatic Society of Bengal* **1872**:124–131.
- Strimmer K, Rambaut A. 2002.** Inferring confidence sets of possibly misspecified gene trees. *Proceedings of the Royal Society B: Biological Sciences* **269**:137–142 DOI [10.1098/rspb.2001.1862](https://doi.org/10.1098/rspb.2001.1862).
- Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004.** Status and trends of amphibian declines and extinctions worldwide. *Science* **306**:1783–1786 DOI [10.1126/science.1103538](https://doi.org/10.1126/science.1103538).
- Su T, Farnsworth A, Spicer RA, Huang J, Wu F-X, Liu J, Li S-F, Xing Y-W, Huang Y-J, Deng W-Y-D, Tang H, Xu C-L, Zhao F, Srivastava G, Valdes PJ, Deng T, Zhou Z-K. 2019.** No high Tibetan Plateau until the Neogene. *Science Advances* **5**:eaav2189 DOI [10.1126/sciadv.aav2189](https://doi.org/10.1126/sciadv.aav2189).
- Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. 2018.** Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution* **4**:vey016.
- Sun YB, Fu TT, Jin JQ, Murphy RW, Hillis DM, Zhang YP, Che J. 2018.** Species groups distributed across elevational gradients reveal convergent and continuous genetic adaptation to high elevations. *Proceedings of the National Academy of Sciences of the United States of America* **115**:E10634–E10641 DOI [10.1073/pnas.1813593115](https://doi.org/10.1073/pnas.1813593115).
- Sun J, Xu Q, Liu WZ, Zhang ZS, Xue L, Zhao P. 2014.** Palynological evidence for the latest Oligocene –early Miocene paleoelevation estimate in the Lunpola Basin, central Tibet. *Palaeogeography, Palaeoclimatology, Palaeoecology* **399**:21–30 DOI [10.1016/j.palaeo.2014.02.004](https://doi.org/10.1016/j.palaeo.2014.02.004).
- Tapponnier P, Xu ZQ, Roger F, Meyer B, Arnaud N, Wittlinger G, Yang JS. 2001.** Oblique stepwise rise and growth of the Tibet Plateau. *Science* **294**:1671–1677 DOI [10.1126/science.105978](https://doi.org/10.1126/science.105978).
- Wagner P, Buaer AM, Leviton AE, Wilms TM, Böhme W. 2016.** A checklist of the amphibians and reptiles of Afghanistan—exploring herpetodiversity using biodiversity archives. *Proceedings of the California Academy of Sciences* **63**:457–565.

- Wang CS, Dai J, Zhao X, Li Y, Graham SA, He DC, Ran B, Meng J. 2014. Outward-growth of the Tibetan Plateau during the Cenozoic: a review. *Tectonics* **621**:1–43.
- Wang C, Zhao X, Liu Z, Lippert PC, Graham SA, Coe RS, Yi H, Zhu L, Liu S, Li Y. 2008. Constraints on the early uplift history of the Tibetan Plateau. *Proceedings of the National Academy of Sciences of the United States of America* **105**:4987–4992 DOI [10.1073/pnas.0703595105](https://doi.org/10.1073/pnas.0703595105).
- Wei Y, Zhang K, Garzzone CN, Xu Y, Song B, Ji J. 2016. Low palaeoelevation of the northern Lhasa terrane during late Eocene: fossil foraminifera and stable isotope evidence from the Gerze Basin. *Scientific Reports* **6**:27508 DOI [10.1038/srep27508](https://doi.org/10.1038/srep27508).
- Wiens JJ. 2011. The niche, biogeography and species interactions. *Philosophical Transactions of the Royal Society B-Biological Sciences* **366**:2336–2350 DOI [10.1098/rstb.2011.0059](https://doi.org/10.1098/rstb.2011.0059).
- Wiens JJ, Sukumaran J, Pyron RA, Brown RM. 2009. Evolutionary and biogeographic origins of high tropical diversity in old world frogs (Ranidae). *Evolution* **63**:1217–1231 DOI [10.1111/j.1558-5646.2009.00610.x](https://doi.org/10.1111/j.1558-5646.2009.00610.x).
- Wu F, Miao D, Chang MM, Shi G, Wang N. 2017. Fossil climbing perch and associated plant megafossils indicate a warm and wet central Tibet during the late Oligocene. *Scientific Reports* **7**:878 DOI [10.1038/s41598-017-00928-9](https://doi.org/10.1038/s41598-017-00928-9).
- Xie W, Lewis PO, Fan Y, Kuo L, Chen M-H. 2011. Improving marginal likelihood estimation for bayesian phylogenetic model selection. *Systematic Biology* **60**:150–160 DOI [10.1093/sysbio/syq085](https://doi.org/10.1093/sysbio/syq085).
- Xu Q, Ding L, Zhang L, Cai F, Lai Q, Yang DT, Liu-Zheng J. 2013. Paleogene high elevations in the Qiangtang Terrane, central Tibetan Plateau. *Earth and Planetary Science Letters* **362**:31–42 DOI [10.1016/j.epsl.2012.11.058](https://doi.org/10.1016/j.epsl.2012.11.058).
- Xu YD, Zhang KX, Wang GC, Jiang SS, Chen FN, Xiang SY, Dupont-Nivet G, Hoorn C. 2012. Extended stratigraphy, palynology and depositional environments record the initiation of the Himalayan Gyirong Basin (Neogene China). *Journal of Southeast Asian Earth Sciences* **44**:77–93 DOI [10.1016/j.jseaes.2011.04.007](https://doi.org/10.1016/j.jseaes.2011.04.007).
- Zhang R, Jiang DB, Ramstein G, Zhang ZS, Lippert PC, Yu E. 2018. Changes in Tibetan Plateau latitude as an important factor for understanding East Asian climate since the Eocene: a modeling study. *Earth and Planetary Science Letters* **484**:295–308 DOI [10.1016/j.epsl.2017.12.034](https://doi.org/10.1016/j.epsl.2017.12.034).
- Zhao W-L, Morgan WJ. 1985. Uplift of Tibetan Plateau. *Tectonics* **4**:359–369 DOI [10.1029/TC004i004p00359](https://doi.org/10.1029/TC004i004p00359).