Species Delimitation and evolutionary history of tree frogs in the Hyla chinensis-group (Hylidae, Amphibian)

Tao Pan  
Anhui Normal University

Guiyou Wu  
Anhui University

Xing Kang  
Anhui University

Peng Yan  
Anhui Normal University

Izaz Ali  
Anhui Normal University

Wenliang Zhou  
Anhui University

Jiatang Li  
Chinese Academy of Sciences

Xiaobing Wu  
Anhui Normal University  
https://orcid.org/0000-0002-6690-3822

Baowei Zhang  
Anhui University

Research article

Keywords: Hyla chinensis-group, Species delimitation, Phylogeny, Biogeography, evolutionary history

Posted Date: September 8th, 2019

DOI: https://doi.org/10.21203/rs.2.14113/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

Background Species are the cornerstone in many domains of biology research, which made the accurate species delimitation became critically important. In this study, the systematics and biogeography of the Hyla chinensis-group were analyzed based on phylogeny, species delimitation and ancestral area reconstruction methods.

Results The phylogenetic results showed six specific clusters existed in the H. chinensis-group. BPP analysis indicated that six distinct species exist due to the high probability values (>0.95), which were also supported by the BF analysis. The divergence time of the H. chinensis-group is estimated to date back to 18.84 Mya in the early Miocene. Combining the results of ancestral area reconstruction, the H. chinensis-group might have originated from Guangxi-Hainan, then spread eastwardly and reached Nanling mountains, Wuyi mountains, Huangshan mountains and Taiwan. In rightabout colonization, it is gradually extended to the Yunnan-Guizhou Plateau, Sichuan basin, Qinling mountains and Dabie mountains. Considering the geological movement from early Miocene to Pliocene, the colonization pattern of the H. chinensis-group maybe closely related to the progressive uplift of Qinghai-Tibetan Plateau (QTP) and historical climate change.

Conclusions Our study provides evidence for species delimitation and speciation process within the H. chinensis-group. Our study supports the hypothesis that the evolutionary divergence in this species group was a consequence of the progressive uplift of QTP and environmental change.

Introduction

For biogeography, abiotic factors (e.g. climate changes and tectonic events), and biological factors (e.g. interspecific or intraspecific interactions, competition and predation) acted as the major drivers temporally and geographically for biological evolution and diversification[1]. Generally, for mountainous landscapes, the interactions of those factors provided beneficial conditions for the various microhabitats. Herein, those species endemic to mountain habitats often exhibit special phylogeographic pattern, such as the relatively small populations with well-defined geographical boundaries [2–4]. In the southern China, many mountains (e.g., Hengduan Mountains, Qinling Mountains, Daba Mountains, Wuyi Mountains, Dabie Mountains) are scattered, which form potential spatially isolated sky islands, providing various microhabitats with beneficial conditions for the speciation process of endemic species [5, 6]. For example, due to the various microhabitats under climate and tectonic events, the Qinghai-Tibetan Plateau (QTP) had significant influence on the evolution of many animal groups[7, 8].

Species are considered as a cornerstone of research in biology fields (e.g. ecology, conservation biology, evolutionary biology, biogeography) [9]. For the effective biological studies, appropriate and accurate species delimitation is becoming increasingly urgent [10–15]. The genus Hyla (Hylidae, Anura) comprised 35 recent described species (19 species distribute in Eurasia; 16 species distributed in North and Central America)[16, 17]. H. chinensis-group, mainly distributed in China, was one of the species complexes in
As for the number of species identified in the *H. chinensis* group, it is controversial\cite{17, 18}. One supported that it included 7 species (*H. annectans, H. chinensis, H. hallowelli, H. sanchiangensis, H. simplex, H. tsinlingensis*, and *H. zhaopingensis*)\cite{18}; the other study supported only 6 species (*H. annectans, H. chinensis, H. simplex, H. sanchiangensis, H. tsinlingensis*, and *H. zhaopingensis*) and five subspecies in *H. annectans* (*H. a. chuanxiensis, H. a. gongshanensis, H. a. jingdongensis, H. a. tengchongensis*, and *H. a. wulingensis*)\cite{17}. Combined those results, it is more urgent to solve the problem of determined number of species and subspecies within this species complex based on species delimitation methods. On the other hand, Li *et al.* (2015) had demonstrated that the *Hyla* originated from North America, then diffused to China via Beringia during the Middle Eocene to Early Oligocene\cite{19, 20}, which may be inferred that the speciation of *H. chinensis*-group may from northern China to the southern China. However, the phylogenetic tree in Li *et al.*, (2015) studies disclosed the base clades of *H. chinensis*-group were all located in the southern China, which maybe hint another expansion route of the *H. chinensis*-group.

Using genetic data and multiple analyses methods, to solve taxonomic uncertainties enable us to disclose phylogenetic topology and speciation process. Here, we reveal a phylogeny of the *H. chinensis*-group based on multiple mitochondrial and nuclear gene covering of currently described species or subspecies within the *H. chinensis*-group\cite{17}. On the basis of species delimitation methods, we aim to clarify systematic and taxonomic matters bound up with species within the *H. chinensis*-group, meanwhile, we evaluate whether orogeny and climate oscillations affected the speciation and evolutionary history of *H. chinensis*-group.

**Methods**

**Ethics statements**

In this study, the sample collection of *H. tsinlingensis* and *H. chinensis* was conducted by a long-term investigation project on amphibians diversity in Dabie Mountains and Huangshan Mountains. This investigation project and sample collection were approved by Anhui Normal University Academic Ethics Committee, Anhui Province, China.

**Taxon sampling**

Based on previous study, we embraced almost all currently recognized species (76 individuals) within the *H. chinensis*-group \cite{17}and choose two species (*H. arborea, H. orientalis*) as outgroups. Additionally, our own specimens (17 *H. tsinlingensis* individuals and 2 *H. chinensis* individuals) were collected from Dabie Mountains and Huangshan Mountains during 2011 to 2014, all samples were non-invasive sampling and the specimens were stored in School of Life Sciences, Anhui University, China (Fig.1). Details on specimen vouchers and GenBank accession number, and specimens sites are listed in Table S1.
Laboratory methods

The proteinase K digestion and phenol/chloroform extraction method were used to extracted total genomic DNA [21]. For combined previous sequence data[17], the same genes were selected based on published primers and new primers (Table S2), including four mitochondrial genes (12S ribosomal small subunit gene/12S rRNA; NADH dehydrogenase subunit 1 gene/ND1, tRNA-Leu and the partial 16S ribosomal large subunit gene/16S) and one nuclear protein-coding gene (proopiomelanocortin A/POMC) [22].

All PCRs were performed within the same conditions in 30 μl volume: 10 to 40 ng of genomic DNA, 15 μl 2×EasyTaq PCR SuperMix polymerase (containing 1U Ex Taq, 0.4mM dNTP, 3mM Mg²⁺, TransGen Biotech) and 0.2 μM of primers. Polymerase chain reaction (PCR) reactions were performed by the following protocol: an initial denaturing step of 5 min at 94°C, followed by 32 cycles with denaturing 30 s at 94°C, annealing 30 s at 50°C and 55°C (for mitochondrial gene and nuclear gene, respectively), extending 40 s and 100 s (for mitochondrial gene and nuclear gene, respectively) at 72°C, and a final extension step of 10 min conducted at 72°C. PCR samples were checked on a 1% agarose gel. Subsequently, PCR products were purified by EasyPure PCR Purification Kit (TransGene) and each fragment was sequenced in both directions on the ABI 3730 semi-automated Sequencer (PE Applied Biosystems).

Sequence processing and phylogenetic analyses

The DNA analysis package DNASTAR Lasergene Seqman and EditSeq 7.1 were used to proofread or assemble the resulting sequences of all genes[23] with default parameters, and the nucleotide sequences were checked by eyes. All the genes were concatenated for analysis and aligned in MEGA 6.0 [24]. Aligned sequences had a total length of 2474 bp (12S rRNA, 815 bp; 16S+tRNA+ND1, 1172bp; POMC, 487 bp). Two datasets were applied in phylogenetic analyses: (1) a data set consisting of the combined mtDNA genes (12S rRNA+16S+tRNA+ND1) was used to conducted species tree, Bayes factor delimitation (BFD) analyses[25], infer divergence times, phylogenetic network and genetic distance analysis; (2) the entire set of mitochondrial and nuclear genes (12S rRNA+16S+tRNA+ND1+POMC) was used to conducted the phylogenetic reconstruction (maximum likelihood, ML; Bayesian) and Bayesian Phylogenetics and Phylogeography (BPP) analysis[15, 26].

Before phylogenetic analysis, the software jModeltest v.2[27] was used to find the best-fit nucleotide substitution model of each gene using Bayesian information criterion (BIC), and these optimal model (GTR+G, 12S; GTR+I+G, 16S+tRNA+ND1+POMC) were selected and implemented in all downstream analysis. Bayesian phylogenetic analysis was performed on different partitions of mitochondrial and nuclear datasets with a mixed-model approach separated into using MrBayes v.3.2.2 [28]. The homologous sequence of H. arborea and H. orientalis was used as outgroups. Two independent runs of Markov Chain Monte Carlo (MCMC) analyses for 10 million generations were conducted. The run was
sampled every 1,000 generations and 10% of the initial samples were discarded as “burn-in “. The maximum likelihood (ML) tree was generated with RAxML v.7.0.3 [29] using the GTR model for mitochondrial and nuclear datasets. Support of nodes was calculated with 1000 bootstrap replicates with the fast bootstrapping algorithm. Aside from the above analysis, we also operated 'net between putative species mean distance' between the H. chinensis-group species at mitochondrial genes in MEGA.

Divergence time estimation

Mitochondrial genes were used to estimate divergence times among H. chinensis-group in BEAST v.1.8.0 [30]. A MCMC approach with uncorrelated lognormal relax molecular clock for rate variation was set. Two independent runs were performed, consisting of 10 million generations, each run sampling every 1000 generations with a burn-in set to 10% of the samples. Tracer 1.6 were used to check the stationarity of results [31]. TreeAnnotator v.1.8.0 [31]and FigTree v. 1.4.2 [32] was used to annotate and visualize tree information. In the absence of appropriate fossils, we selected several calibration points information from previous work[17], assuming a normal distribution for the divergence time between H. arborea-group and the H. chinensis-group, with a mean of 23 millions of years ago (Mya) and standard deviation of 3.04 (thus effectively spanning a large range from 18 to 28 Mya).

Bayes factor delimitation (BFD)

The Bayes factor (BF) is a common species delimitation selection tool in phylogenetics [25] based on the marginal-likelihood estimates (MLE) via path-sampling (PS) and stepping-stone sampling (SS) analyses [33–35]. The scopes of BF are as follows: 0 < BF < 2 is not worth more than a bare mention, 2 < BF < 6 is positive evidence, 6 < BF < 10 is strong support, and BF > 10 is decisive [30]. Coupled with the former studies[17, 18] and the above phylogenetic analyses inference, six ingroup species in the H. chinensis-group were assumed and four species delimitation scenarios(True, Lump, Split and Reassign) were generated to disclose the inner species number in *BEAST [36]. For “True” scenario, individuals were assigned to six ingroup species in the H. chinensis-group as prior set. For the “Lump” scenario, we inferred that two ingroup species were regarded as a single species, corresponding to the ingroup number of species from six to five. In contrast, the “Split” scenario suggested two ingroup species each split into two species, which indicated the total number of ingroup species from six to eight. As to the “Reassign” scenario, a total of three individuals were “incorrectly” reassigned to different ingroup species than in the “True” tree. PS and SS analyses were each run for totaling $10^8$ generations with a chain length of $10^6$ generations for 100 path steps.

Bayesian Phylogenetics and Phylogeography (BPP)
Contrast to the results of our BFD method to a commonly used method of species delimitation, we performed species delimitation analysis with the phased dataset for the two mitochondrial loci and one nuclear locus implemented in BPP v.3.0 [15, 26]. This method utilizes a reversible jump Markov chain Monte Carlo (rjMCMC) algorithm to calculate the posterior probabilities to speciation events that contain more or less lineages [15]. Between all BPP analyses, probability values ≥ 0.95 were considered as strong support in favor of a speciation event [37]. The guide tree was generated from the species tree.

The prior of ancestral population size (θ) and root age (τ) are directly related to the posterior probabilities of each results for models. For example, the combination of large values for θ and small values for τ is assumed to be the most conservative, leading to a lower number of speciation events[15, 37]). We evaluated three schemes of the prior of the θ and τ: (1) θ = G (1:2000) and τ = G (1:10), (2) θ = G (1:2000) and τ = G (1:100), (3) θ = G (2:2000) and τ = G (1:10). The parameters of the rjMCMC analyses were set as500,000 generations with sampling every 50 step, and 100,000 burn-in steps.

Species tree inference

The coalescent-based method implemented in *BEAST was used to construct the species tree[36]based on mitochondrial genes. Two independent runs of 20 million generations with were conducted. The sample frequency was set as 10,000 generations and 10% of the total samples were discarded as burn-in. The other models and prior specification applied were set as follows: the nucleotide substitution model: HKY; Relaxed Uncorrelated Lognormal Clock (estimate); Yule process of speciation; random starting tree; alpha Uniform (0, 10). The convergence was checked by examining trace plots and histograms in Tracer. Runs were combined using LogCombiner. In addition, we tended to construct a uncorrected p-distances phylogenetic network with heterozygous ambiguities averaged and normalized by Splitstree v. 4.13.1 [38].The neighbor-net ordinary least squares variance and equal angle algorithm were used and 1,000 bootstrap replicates were calculated to assess branch support.

Ancestral area reconstruction

Geographical regions were delimited in terms of the current distribution area of the sequenced species of the *H. chinensis*-group, at the same time, the information coming from the relevant literatures[16, 39–41]. The five areas were: N, the southern China (Guangxi-Hainan provinces), which is a main distribution area of *H. zhaopingensis*; W, Eastern China; S, the southern Guangxi province in China, which is an important distribution range about *H. sanchiangensis*; Y, the eastern of the Tibetan Plateau (Yunnan-Guizhou Plateau and Sichuan basin); Z, the Tsinling-Dabie Mountains(Fig.1, 2). Taking the effect of the LAGRANGE model components into account, we designed experiments that transform the adjacency matrix, hence, resulting in a total of 3 experiments (M0, M1, M2). This is according to the assumption that the *H. chinensis*-group, like all organisms, have a lower possibility to disperse over non-adjacent areas than adjacent areas. For this reason, no ranges were forbidden for M0; CD, SD and ND were forbidden for
M1; CD, ND, SD and NY were forbidden for M2. To select the optimal model, we compared their log-likelihood (the data presented by LAGRANGE), meanwhile, used the standard cut-off value of two log-likelihood units as indicating a conspicuous imbalance between models, with the less negative likelihood being preferred [42]. Ancestral areas were reconstructed by dispersal-extinction-cladogenesis model [43] as carried out in the software Lagrange v20130526 [44], and the chronogram obtained in BEAST was the starting component of the analyses.

**Results**

Phylogenetic analysis of concatenated sequences (mtDNA data and nuclear gene) recovered a well-resolved tree with six major clades (labeled A to F) within the *H. chinensis*-group (Fig. 2 and Fig. S1). Clade A corresponds to *H. tsinlingensis* and *Hyla annectans chuanxiensis*, which mainly located in the Qinling-Dabie mountains; Clade B included *H. annectans, H. a. wulingensis* and *H. a. jingdongensis*; while *H. a. gongshanensis* and *H. a. tengchongensis* within clade C, and they are all distributed in the Yunnan-Guizhou Plateau and Sichuan basin; The remaining clade D (i.e., *H. sanchiangensis*), E (i.e., *H. chinensis*) and F (i.e., *H. zhaopingensis*) located in the Guangxi province, Hainan province and the Eastern China, respectively (Fig. 2 and Fig. S1). The phylogenetic network of *H. chinensis*-group showed the consistent groupings compared with the phylogenetic methods (Fig. 3). Dating analyses indicated that the most recent common ancestor (MRCA) of the *H. chinensis*-group dates back to 18.84 Mya (95% of the highest posterior density [HPD], 19.50–17.18 Mya) in the mid-Miocene. The divergence time between clades within *H. chinensis*-group was taken place from late-Miocene (11.88 Mya) to the early Pleistocene (around 4.82 Mya) (Fig.2).

The BFD based on PS (BF, 12.62) and SS (BF, 20.84) models decisive supported six species in the *H. chinensis*-group (Table 1), corresponding to the six clades disclosed by phylogenetic tree (Fig. 2 and Fig. S1). BPP methods also supported six separated species due to higher than 0.95 probability values (Table 2). Species tree, consistent with BPP tree topology, recovered a concordant, robust phylogenetic topology (Fig. 4). Pairwise sequence divergence (p uncorrected distance) between hidden species in *H. chinensis*-group ranged from 2.1% (A vs B) to 11.4% (E vs F) (Table 3).

In the ancestral area reconstruction, the best model for the *H. chinensis*-group was M2, which supported that it was dispersed from southern China to the Qinling-Dabie mountains and to the Eastern of the Tibetan Plateau were restricted (Table 4). The analyses supported that the southern China (Guangxi-Hainan provinces, Area N) and Eastern China (Area W) as the ancestral area of *H. chinensis*-group and most speciation events were attributed to the progressive uplift of the Himalayas (Fig. 2 and Table S3). Additionally, the *H. tsinlingensis* was originated from the Eastern of the Tibetan Plateau (Yunnan-Guizhou Plateau and Sichuan basin, Area Y).

**Discussion**
The phylogenetic analysis identified all the individuals of the *H. chinensis*-group formed into six genetically distinct population clusters (i.e., Clade A–F) (Fig. 2, 3 and Fig. S1). Based on BF and BPP methods, the species delimitation suggested these six genetically distinct clades could be regarded as hidden separated species in the *H. chinensis*-group, which also got the supported from genetic distance (Table 3). In brief, clade A corresponds to *H. tsinlingensis* and *Hyla annectans chuanxiensis*; clade B included *H. annectans*, *H. a. wulingensis* and *H. a. jingdongensis*; while *H. a. gongshanensis* and *H. a. tengchongensis* within clade C; The remaining clades (D, E, and F) corresponding to *H. sanchiangensis*, *H. chinensis* and *H. zhaopingensis*, respectively (Fig. 2 and Fig. S1). Compared with the study of Li et al. (2015), some minor difference exists: *Hyla annectans chuanxiensis* belong to *H. tsinlingensis*, not *H. annectans*; two sub-species of *H. annectans* (*H. a. gongshanensis* and *H. a. tengchongensis*) regarded as separated species.

The dated phylogenetic tree indicated the Clade F (about 18.84 Ma) is at the base of *H. chinensis*-group and contains *H. zhaopingensis* in Southern China (Guangxi province). Six putative hidden species in *H. chinensis*-group (Clade A to F) approximately correspond to the three areas of China: the Eastern of Qinghai-Tibetan Plateau (i.e. the Yunnan-Guizhou Plateau and Sichuan basin), the Eastern and Southern China and the Qinling-Dabie mountains. Additionally, the first stage of speciation (e.g. split between Clade D and E) in *H. chinensis*-group occurs in Southern and Eastern in China during Middle Miocene (ca 18–10 Ma). *Hyla* is a small, arboreal and semi-aquatic frog, prefer to live in warm and damp environment, which is widely inhabited in boscage, paddy fields or edges of rivers, breeds in still water in ponds or paddy fields[45]. During this period, the southern China humid climate, conductive to the survival of the species. For example, palaeobotanical data indicated that the south-eastern of the QTP was warm and humid climate, was dominated by subtropical vegetation during the Miocene[46], which had provided an opportunity for the first stage of speciation in *H. chinensis*-group.

The second stage of speciation in *H. chinensis*-group occurs in the Southwest of China (Yunnan Province and Sichuan Province) and the Qinling Mountains-Dabie Mountains in China from the late Miocene to Pliocene (5.57 ~ 4.82 Ma). During the Late Miocene to Pliocene, the progressive uplift of the QTP particularly at its eastern and northern margin (mostly province of Yunnan, Sichuan and Qinghai), led to the formation of some rivers, the Hengduanshan hotspot of biodiversity was composed by those areas[7]. In addition, the upheaval of the QTP had a significant impact on the atmospheric circulation in Asia and promoted the development of the Asian monsoon system[47, 48]. The East Asian Monsoon system was controlled China’s climate at that time, and this condition brought moisture air from the ocean to East China[49]. Combined the geological events, they may contributed to the second stage of speciation in *H. chinensis*-group.

In conclusion, the rapid uplifting mountain ranges (the Tibetan Plateau and its adjacent mountain) formed a blocky orographic barrier for many endemic species [7], which also played an important role in the formation Asian monsoon system [47, 50, 51]. Additionally, three East Asian monsoon intensification periods (~15 Ma, ~8 Ma and 4–3 Ma)[46, 52, 53] also had urged the formation of humid and warm climate in south China [54], which was favorable for geographical dispersal, especially for amphibian
More dispersal events often means that these species had more opportunities for allopatric divergence, which greatly affected the high levels of inter-population genetic divergence and unique patterns of genetic structure[7, 55–58]. Therefore, based on those results, we can inferred that the speciation and diffusion in the *H. chinensis*-group had been from Guangxi-Hainan provinces to Guangxi province and Eastern China, and then to the Yunnan-Guizhou Plateau and Sichuan basin, finally spread to Qinling-Dabie Mountains. The diversification and speciation in the *H. chinensis*-group also may be related to the special geological deformations and the climatic history.

**Conclusion**

As one of the species complexes in *Hyla*, the determined species number in *H. chinensis*-group was full of competing. Until now, no research focus on the species delimitation based on the genetic data. In this study, different species delimitation approaches revealed that multiple species exist in the *H. chinensis*-group. These methods indicated that there are six distinct species (from Clade A-F respectively) in this species group. The progressive uplift of QTP and climate change led to the dispersal progress and formation of hidden species diversity in the *Hyla chinensis*-group. Nevertheless, for providing the integrative revision of this species group, diagnostic morphological characters and other ecological evidences are still needed to supplied based on thorough quantitative multivariate analysis.

**Declarations**

**Ethics.** In the present study, our experimental procedures and sample collection complied with the current laws on animal welfare and research in China, and were specifically approved by the Animal Research Ethics Committee of Anhui Normal University.

**Data accessibility.** Data for all analyses reported in this paper can be publicly accessible in NCBI after the acceptance of our manuscript. The accession number of GenBank will be added after the article is accepted.

**Authors’ contributions.** B. W.Z, J. T. L. and X. B. W. conceived the study; T. P., G. Y. W., X. K., P. Y. and W. L. Z. contributed to sample collection; T. P., G. Y. W., X. K., P. Y., I. A. and W. L. Z. carried out laboratory work; T. P., G. Y. W. and X. K. analyzed the data and wrote the paper with contributions from B. W.Z, X. B. W., J. T. L. and T. P. The language was corrected by I. A. All authors approved the final version of the manuscript and agree to be held accountable for its content.

**Competing interests.** We declare we have no competing interests.

**Funding.** This work is supported by Anhui Natural Science Foundation (Youth, 1908085QC127), 2018 Funding for research activities of post-doctoral researchers in Anhui Province in the design of study, field work, sample collection, lab work and data analysis. The writing and publishing were supported by Anhui Province Academic and Technical Leader & Backup Candidate Academic Research Activities Fund (2017H130).
Acknowledgements. We are grateful to Chaochao Hu, Lifu Qian, Ke Fang, Chengcheng Wang, Yanan Zhang, and Xiaonan Sun for their help in the wild survey and data analysis.

References

1. Benton MJ: The Red Queen and the Court Jester: species diversity and the role of biotic and abiotic factors through time. Science 2009, 323(5915):728–732.

2. Huang ZS, Yu FL, Gong HS, Song YL, Zeng ZG, Zhang Q: Phylogeographical structure and demographic expansion in the endemic alpine stream salamander (Hynobiidae: Batrachuperus) of the Qinling Mountains. Sci Rep 2017, 7(1871):1–13.

3. Shepard DB, Burbrink FT: Local-scale environmental variation generates highly divergent lineages associated with stream drainages in a terrestrial salamander, Plethodon caddoensis. Mol Phylogenet Evol 2011, 59(2):399–411.

4. Pan T, Sun ZL, Lai XL, Orozcoterwengeld P, Yan P, Wu GY, Wang H, Zhu WQ, Wu XB, Zhang BW: Hidden species diversity in Pachyhynobius: A multiple approaches species delimitation with mitogenomes. Mol Phylogenet Evol 2019, 137(2019):138–145.

5. Gao Y, Ai B, Kong HH, Kang M, Huang HW: Geographical pattern of isolation and diversification in karst habitat islands: a case study in the Primulina eburnea complex. J Biogeogr 2015, 42(11):2131–2144.

6. Zhen Y, Chen PP, Bu WJ: Terrestrial mountain islands and Pleistocene climate fluctuations as motors for speciation: a case study on the genus Pseudovelia (Hemiptera: Veliidae). Sci Rep 2016, 6.33625.

7. Favre A, Päckert M, Pauls SU, Jähnig SC, Uhl D, Michalak I, Muellner-Riehl AN: The role of the uplift of the Qinghai-Tibetan Plateau for the evolution of Tibetan biotas. Biol Rev 2015, 90(1):236–253.

8. Päckert M, Martens J, Sun YH, Severinghaus LL, Nazarenko AA, Ting J, Töpfer T, Tietze DT: Horizontal and elevational phylogeographic patterns of Himalayan and Southeast Asian forest passerines (Aves: Passeriformes). J Biogeogr 2012, 39(3):556–573.

9. Aldhebiani AY: Species concept and speciation. Saudi J Biol Sci 2018, 25(3):437–440.

10. Blair C, Bryson JR: Cryptic diversity and discordance in single-locus species delimitation methods within horned lizards (Phrynosomatidae: Phrynosoma). Mol Ecol Res 2017, 17(6):1–15.

11. Grummer JA, Bryson RW, Reeder TW: Species delimitation using Bayes factors: simulations and application to the Sceloporus scalaris species group (Squamata: Phrynosomatidae). Sys Biol 2014, 63(2):119–133.

12. Kajtoch Ł, Montagna M, Wanat M: Species delimitation within the Bothryorrhynchapion weevils: Multiple evidence from genetics, morphology and ecological associations. Mol Phylogenet Evol 2017,
120:354–363.

13. Kotsakiozi P, Jablonski D, Ilgaz Ç, Kumluças Y, Avci A, Meiri S, Itescu Y, Kukushkin O, Gvoždík V, Scillitani G: Multilocus phylogeny and coalescent species delimitation in Kotsch’s gecko, Mediodactylus kotschyi: hidden diversity and cryptic species. Mol Phylogenet Evol 2018, 125:177–187.

14. Sheridan JA, Stuart BL: Hidden species diversity in Sylvirana nigrovittata (Amphibia: Ranidae) highlights the importance of taxonomic revisions in biodiversity conservation. PLoS One 2018, 13(3):e0192766.

15. Yang ZH, Rannala B: Bayesian species delimitation using multilocus sequence data. P Natl Acad Sci USA 2010, 107(20):9264–9269.

16. Frost DR: Amphibian species of the world: an online reference. Version 6.0. New York: American Museum of Natural History. In.; 2014.

17. Li JT, Wang JS, Nian HH, Litvinchuk SN, Wang JC, Li Y, Rao DQ, Klaus S: Amphibians crossing the Bering Land Bridge: Evidence from holarctic treefrogs (Hyla, Hylidae, Anura). Mol Phylogenet Evol 2015, 87:80–90.

18. Hua X, Fu CZ, Li JT, de Oca ANM, Wiens JJ: A revised phylogeny of Holarctic treefrogs (genus Hyla) based on nuclear and mitochondrial DNA sequences. Herpetologica 2009, 65(3):246–259.

19. Smith SA, Stephens PR, Wiens JJ: Replicate patterns of species richness, historical biogeography, and phylogeny in Holarctic treefrogs. Evolution 2005, 59(11):2433–2450.

20. Wiens JJ, Graham CH, Moen DS, Smith SA, Reeder TW: Evolutionary and ecological causes of the latitudinal diversity gradient in hylid frogs: treefrog trees unearth the roots of high tropical diversity. Am Nat 2006, 168(5):579–596.

21. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning, vol. 2: Cold spring harbor laboratory press New York; 1989.

22. Wiens JJ, Fetzner JW, Parkinson CL, Reeder TW: Hylid frog phylogeny and sampling strategies for speciose clades. Syst Biol 2005, 54(5):778–807.

23. Burland TG: DNASTAR's Lasergene sequence analysis software. In: Bioinformatics Methods and Protocols. Springer; 1999: 71–91.

24. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011, 28(10):2731–2739.

25. Sullivan J, Joyce P: Model selection in phylogenetics. Annu Rev Ecol Evol S 2005, 36(36):445–466.
26. Rannala B, Yang ZH: Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. Genetics 2003, 164(4):1645–1656.

27. Darriba D, Taboada GL, Doallo R, Posada D: JModelTest 2: more models, new heuristics and parallel computing. Nat Methods 2012, 9:772.

28. Ronquist F, Huelsenbeck JP: MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003, 19(12):1572–1574.

29. Stamatakis A: The RAxML 7.0. 3 Manual. Exelixis Lab, Heidelberg Institute for Theoretical Studies, Heidelberg http://www.trex.uqam.ca/documents/RAxML-Manual 2008, 7(3).

30. Drummond AJ, Suchard MA, Xie D, Rambaut A: Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 2012, 29(8):1969–1973.

31. Rambaut A, Drummond AJ: Tracer v1.5. http://beast.bio.ed.ac.uk/Tracer 2007.

32. Rambaut A: FigTree. Version 1.4. 2. http://tree.bio.ed.ac.uk/software/figtree/ 2014.

33. Fan Y, Wu R, Chen MH, Kuo L, Lewis PO: Choosing among partition models in Bayesian phylogenetics. Mol Biol Evol 2011, 28(1):523–532.

34. Li WLS, Drummond AJ: Model averaging and Bayes factor calculation of relaxed molecular clocks in Bayesian phylogenetics. Mol Biol Evol 2012, 29(2):751–761.

35. Xie WG, Lewis PO, Fan Y, Kuo L, Chen MH: Improving marginal likelihood estimation for Bayesian phylogenetic model selection. Syst Biol 2011, 60(2):150.

36. Heled J, Drummond AJ: Bayesian inference of species trees from multilocus data. Mol Biol Evol 2010, 27(3):570–580.

37. Leaché AD, Fujita MK: Bayesian species delimitation in West African forest geckos (Hemidactylus fasciatus). P Roy Soc Lond B Biol 2010, 277(1697):3071–3077.

38. Huson DH, Bryant D: Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 2006, 23(2):254–267.

39. Fei L, Hu SQ, Ye CY, Huang YZ: Fauna Sinica. Amphibia Vol. 2 Anura. In.: Science Press, Beijing; 2009.

40. Li SM, Yang DT: The description of a new subspecies Hyla annectanus gongshanensis from Yunnan. Zool Res 1985, 6(1):23–28.

41. Tang YX, Zhang ZJ: A new species of amphibians from Guangxi. Acta Zootaxonomica Sin 1984, 4:23.
42. Chacón J, Renner SS: *Assessing model sensitivity in ancestral area reconstruction using Lagrange: a case study using the Colchicaceae family*. J Biogeogr 2014, 41(7):1414–1427.

43. Garzione CN, Dettman DL, Quade J, DeCelles PG, Butler RF: *High times on the Tibetan Plateau: Paleoelevation of the Thakkhola graben, Nepal*. Geology 2000, 28(4):339–342.

44. Ree RH, Smith SA: *Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis*. Syst Biol 2008, 57(1):4–14.

45. Fei L: *An illustrated key to Chinese amphibians*: Sichuan Publ. Group; 2005.

46. Jacques FMB, Guo SX, Su T, Xing YW, Huang YJ, Liu YS, Ferguson DK, Zhou ZK: *Quantitative reconstruction of the Late Miocene monsoon climates of southwest China: a case study of the Lincang flora from Yunnan Province*. Palaeogeogr Palaeocl 2011, 304(3):318–327.

47. Tang H, Micheels A, Eronen JT, Ahrens B, Fortelius M: *Asynchronous responses of East Asian and Indian summer monsoons to mountain uplift shown by regional climate modelling experiments*. Clim Dynam 2013, 40(5–6):1531–1549.

48. Kutzbach JE, Prell WL, Ruddiman WF: *Sensitivity of Eurasian climate to surface uplift of the Tibetan Plateau*. J Geol 1993, 101(2):177–190.

49. Liu LP, Eronen JT, Fortelius M: *Significant mid-latitude aridity in the middle Miocene of East Asia*. Palaeogeogr Palaeocl 2009, 279(3):201–206.

50. Guo ZT, Sun B, Zhang ZS, Peng SZ, Xiao GQ, Ge JY, Hao QZ, Qiao YS, Liang MY, Liu JF: *A major reorganization of Asian climate by the early Miocene*. Clim Past 2008, 4(3):153–174.

51. Song JH, Kang HS, Byun YH, Hong SY: *Effects of the Tibetan Plateau on the Asian summer monsoon: a numerical case study using a regional climate model*. Int J Climatol 2010, 30(5):743–759.

52. Molnar P, Boos WR, Battisti DS: *Orographic controls on climate and paleoclimate of Asia: thermal and mechanical roles for the Tibetan Plateau*. Annu Rev Earth Pl Sc 2010, 38(1):77–102.

53. Wan SM, Li AC, Clift PD, Stuut JBW: *Development of the East Asian monsoon: mineralogical and sedimentologic records in the northern South China Sea since 20 Ma*. Palaeogeogr Palaeocl 2007, 254(3):561–582.

54. Sun XG, Wang PX: *How old is the Asian monsoon system?—Palaeobotanical records from China*. Palaeogeogr Palaeocl 2005, 222(3):181–222.

55. Che J, Zhou WW, Hu JS, Yan F, Papenfuss TJ, Wake DB, Zhang YP: *Spiny frogs (Paini) illuminate the history of the Himalayan region and Southeast Asia*. P Natl Acad Sci USA 2010, 107(31):13765–13770.
56. Wu YK, Wang YZ, Jiang K, Hanken J: *Significance of pre-Quaternary climate change for montane species diversity: insights from Asian salamanders (Salamandridae: Pachytriton).* Mol Phylogenet Evol 2013, 66(1):380–390.

57. Yan F, Zhou WW, Zhao HT, Yuan ZY, Wang YY, Jiang K, Jin JQ, Murphy RW, Che J, Zhang YP: *Geological events play a larger role than Pleistocene climatic fluctuations in driving the genetic structure of Quasipaa boulengeri (Anura: Dicroglossidae).* Mol Ecol 2013, 22(4):1120–1133.

58. Ye SP, Huang H, Zheng RQ, Zhang JY, Yang G, Xu SX: *Phylogeographic analyses strongly suggest cryptic speciation in the giant spiny frog (Dicroglossidae: Paa spinosa) and interspecies hybridization in Paa.* PLOS ONE 2013, 8(7):e70403.

59. Zachos J, Pagani M, Sloan L, Thomas E, Billups K: *Trends, rhythms, and aberrations in global climate 65 Ma to present.* Science 2001, 292(5517):686–693.

60. Zachos JC, Dickens GR, Zeebe RE: *An early Cenozoic perspective on greenhouse warming and carbon-cycle dynamics.* Nature 2008, 451(7176):279–283.

**Tables**

**Table 1.** Species delimitation results of the *H. chinensis*-group.

| Model   | Species | MLE Path Sampling (PS) | MLE Stepping Stone (SS) | Rank | BF (PS) | BF (SS) |
|---------|---------|------------------------|-------------------------|------|---------|---------|
| True    | 6       | -7908.64               | -7875.13                | 1    | 12.62   | 20.84   |
| Lump    | 5       | -7966.71               | -7937.86                | 4    | -       | -       |
| Split   | 8       | -7914.95               | -7885.55                | 2    | -       | -       |
| Reassign| 6       | -7964.56               | -7934.90                | 3    | -       | -       |

Note: MLE, Marginal likelihood estimate; BF, Bayes factor; PS, path sampling; SS, stepping stone.

**Table 2** The species delimitation results of the *H. chinensis*-group based on mtDNA and nuclear gene data in BPP.

| Scheme   | Prior distribution | Posterior probabilities |
|----------|--------------------|-------------------------|
|          | θ                  | τ                       |
| Scheme 1 | G (1, 100)         | G (1, 2000)             | 0.98393 |
| Scheme 2 | G (1, 10)          | G (1, 2000)             | 0.98877 |
| Scheme 3 | G (1, 10)          | G (2, 2000)             | 0.98607 |
Table 3. Corrected pairwise genetic distances (%) for mtDNA, among species in six clades of the *chinensis*-group.

| Clade | A      | B      | C      | D    | E    | F    |
|-------|--------|--------|--------|------|------|------|
| A     |        |        |        |      |      |      |
| B     | 0.021  |        |        |      |      |      |
| C     | 0.028  | 0.032  |        |      |      |      |
| D     | 0.075  | 0.075  | 0.072  |      |      |      |
| E     | 0.087  | 0.081  | 0.082  | 0.096|      |      |
| F     | 0.106  | 0.105  | 0.107  | 0.111| 0.114|      |

Table 4. Comparison of different dispersal models in Lagrange. (M0: unconstrained; M1: dispersal from southern China to the Qinling-Dabie mountains were restricted (from C, N, S to D); M2: dispersal from southern China to the Qinling-Dabie mountains and to the eastern of the Tibetan Plateau were restricted (from C, N, S to D and from N to Y).

| Model | -lnL  | Extinction rate | Dispersal rate |
|-------|-------|-----------------|----------------|
| M0    | 20.80 | 5.595e-3        | 4.285e-09      |
| M1    | 20.40 | 6.724e-3        | 4.285e-09      |
| M2    | 20.38 | 8.422e-3        | 4.285e-09      |

Figures
Figure 1

Map with the localities samples of H. chinensis-group in this study. The sampling sites of each clade (A-F) was marked with different triangles or dots in different colors. These clades (A-F) are corresponding to the clades in Fig. 2. The black and white coloration represent elevation.
Figure 2

Chronogram and ancestral reconstructions of the H. chinensis-group. Top panel: time-calibrated phylogeny of the H. chinensis-group based on mitochondrial dataset (The light-blue bars through the nodes indicate 95% HPDs) and ancestral area reconstruction by a dispersal-extinction-cladogenesis model (colored squares), two extensive dispersal events were shown for the origin of N1 and N5 (arrows represent the direction of dispersal), geological sequence of events related to the diversification of H.
chinensis-group including a graphical representation of the extent uplift of TP through time (green shades indicate the portion of the Qinghai-Tibetan Plateau that had achieved altitudes comparable to present day, adapted from[7]). Areas divided for reconstructing ancestral areas are displayed in the top left: N, Southern China (Guangxi-Hainan provinces); W, Eastern China; S, the southern Guangxi province in China; Y, the eastern of the Tibetan Plateau (Yunnan-Guizhou Plateau and Sichuan basin); Z, the Tsinling-Dabie Mountains. Lower panel: temperature changes [59, 60].

Figure 3

Network constructed from the mitochondrial genes of H. chinensis-group based on uncorrected p-distances using SPLITSTREE. The values on nodes indicate bootstrap support (only values above 75% are shown).
Figure 4

Species tree estimated using BEAST based on mitochondrial genes in H. chinensis-group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S1.tif
- TableS1NCBIaccessionnumber.docx
- TableS3Lagrange.docx
- TableS2.docx