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

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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

Hyla. As for the number of species identified in the *H. chinensis* group, it is controversial[17, 18].One supported that it included 7 species (*H. annectans, H. chinensis, H. hallowelli, H. sanchiangensis, H. simplex, H. tsinlingensis,* and *H. zhaopingensis)*[18]; the other study supported only6 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)*[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[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[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 [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, 15μl 2×EasyTaq PCR SuperMix polymerase (containing 1U Ex Taq, 0.4mM dNTP, 3mM Mg^{2+}, TransGen Biotech) and 0.2μM 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 32cycles 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) adata 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 infered 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 frequence 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 carry 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 0.95probability 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 form 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 seperated species.

The dated phylogenetic tree indicated the Clade F (about 18.84 Mya)is at the base of *H. chinensis*-group and contains *H. zhaopingensis* in Southern China (Guangxi province). Six putative hidden speciesin *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 oportunity 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 Mya). 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

[55–58].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 acception 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.

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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	Priordistribution		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	-InL	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

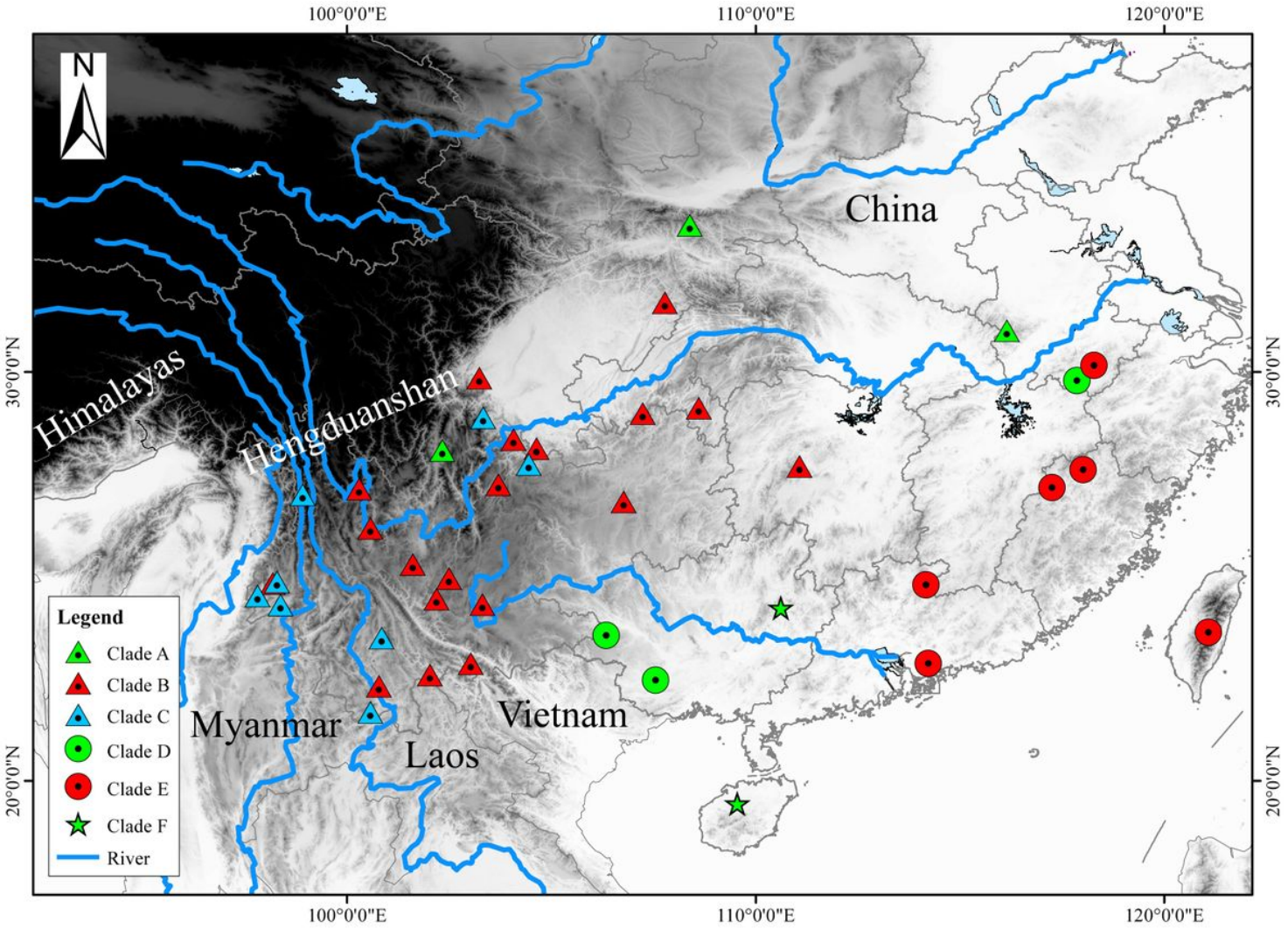

Figure 1

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.

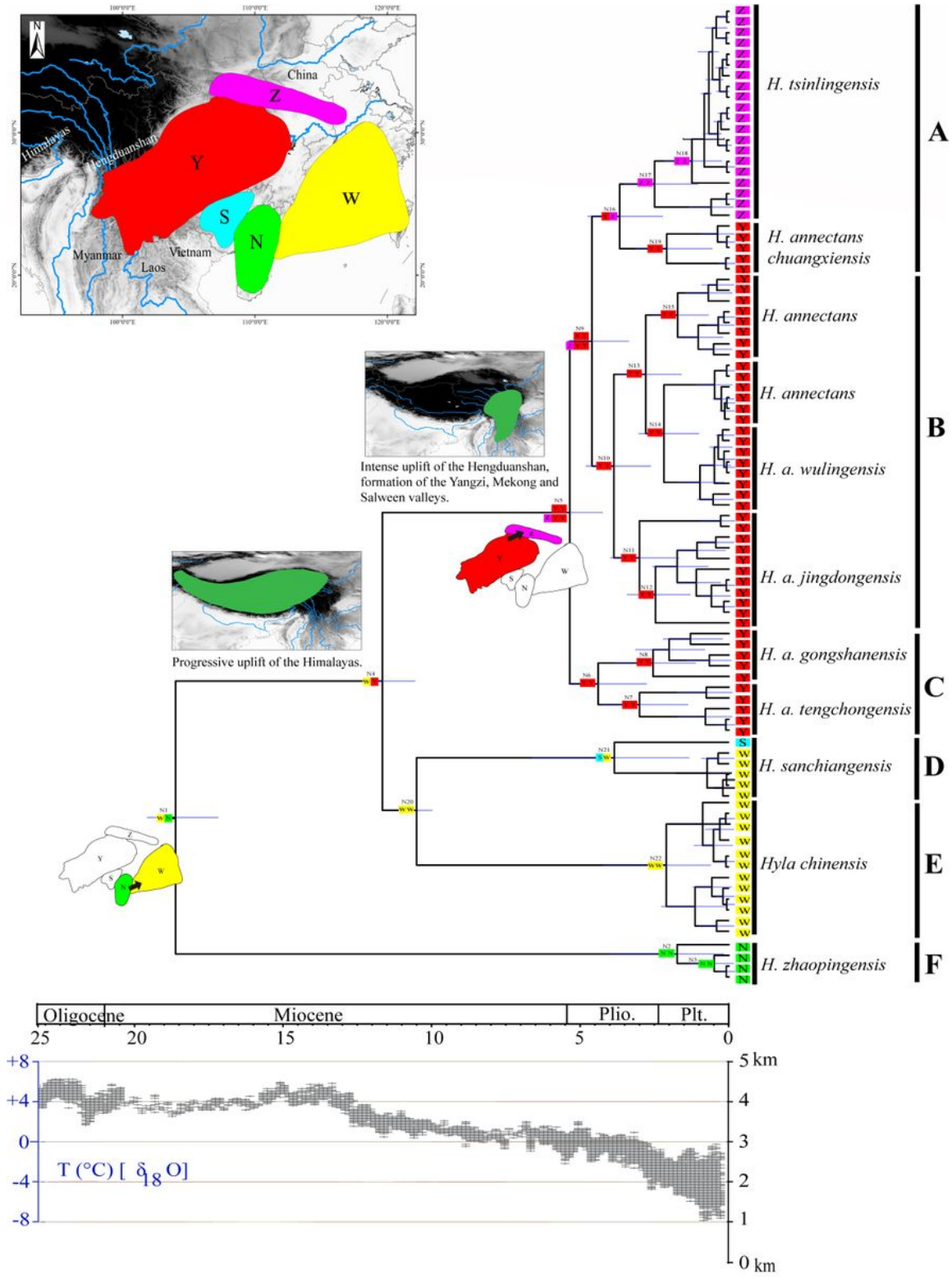

Figure 2

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 TibetanPlateau (Yunnan-Guizhou Plateau and Sichuan basin); Z, the Tsinling-Dabie Mountains. Lower panel: temperature changes [59, 60].

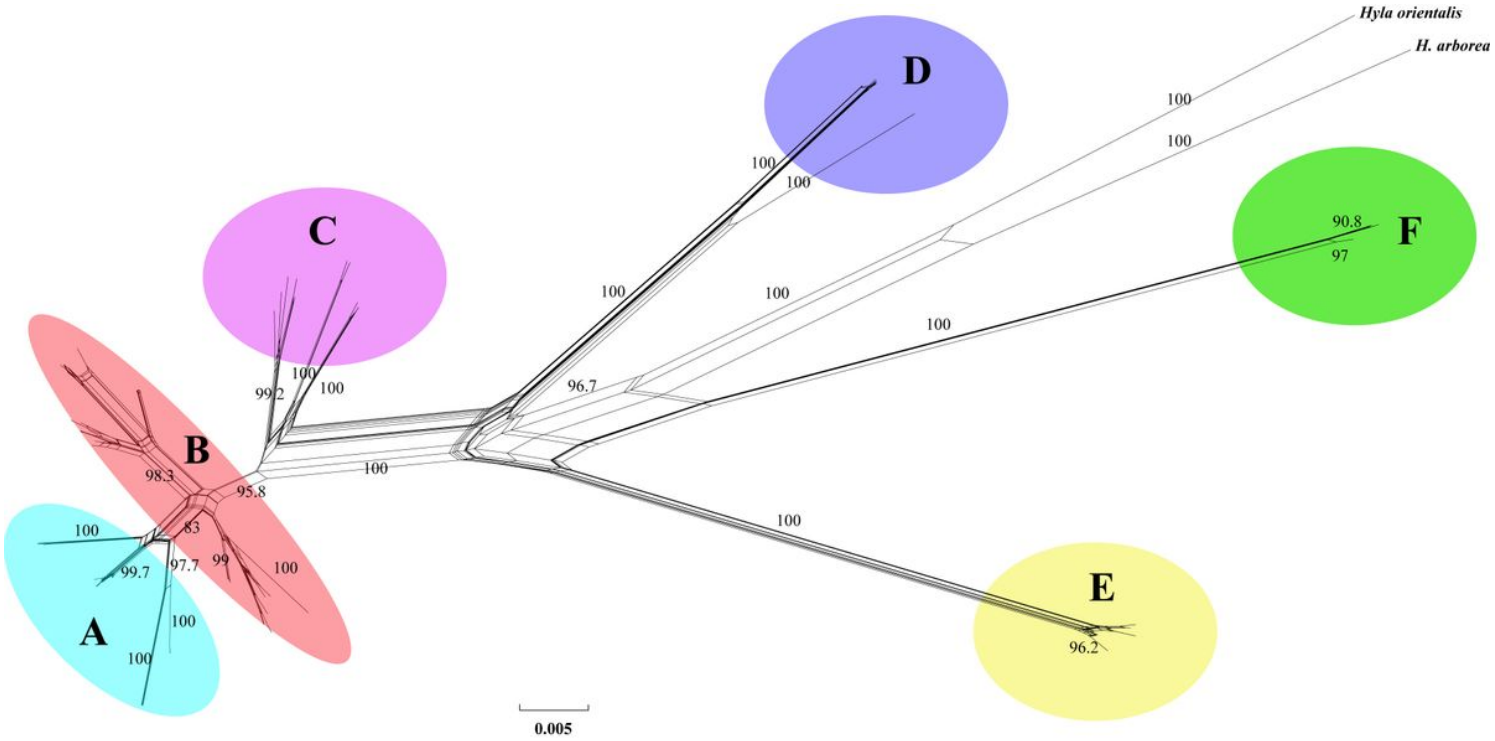

Figure 3

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).

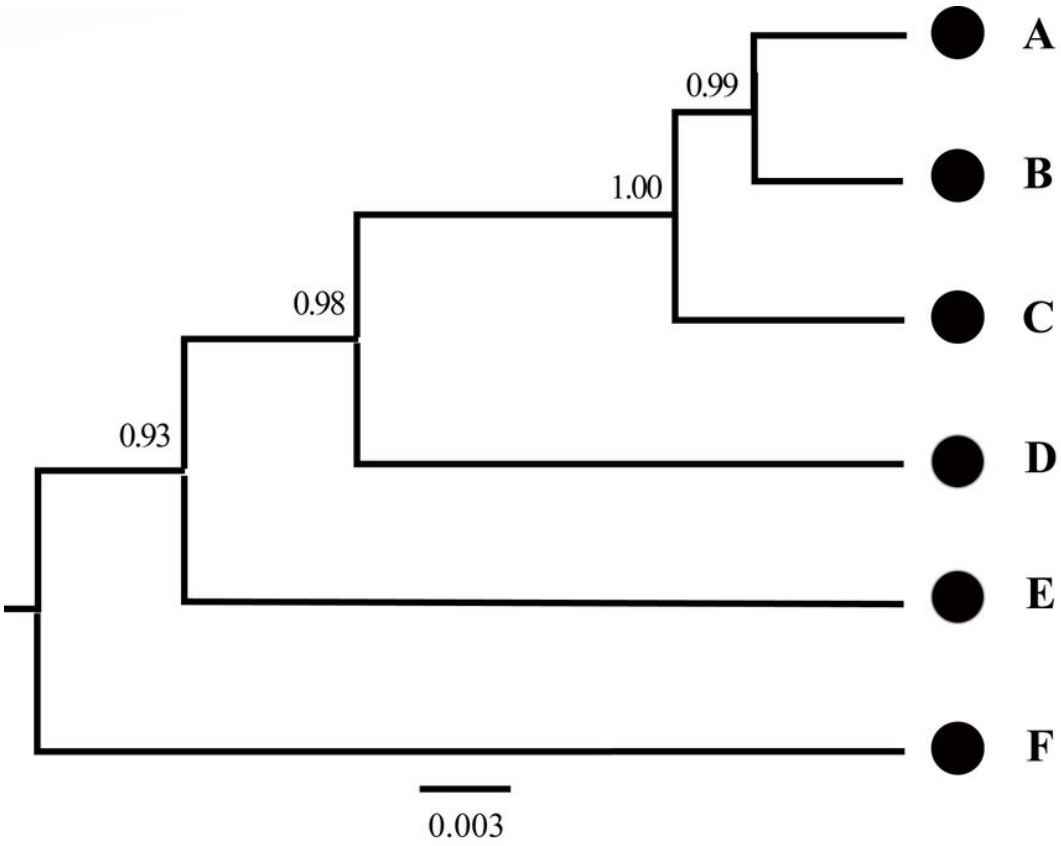

Figure 4

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

Supplementary Files

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