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Species Delimitation and Evolutionary History of Tree Frogs in the *Hyla chinensis* Group (Hylidae, Amphibian)

Peng Yan[1†], Tao Pan[1†], Guiyou Wu[2], Xing Kang[2], Izaz Ali[1], Wenliang Zhou[3], Jiatang Li[4], Xiaobing Wu[1*] and Baowei Zhang[1,2*]

[1] Anhui Provincial Key Laboratory of the Conservation and Exploitation of Biological Resources, College of Life Sciences, Anhui Normal University, Wuhu, China, [2] School of Life Sciences, Anhui University, Hefei, China, [3] Key Lab of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, [4] Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China

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***Correspondence:**
Xiaobing Wu
wuxb@ahnu.edu.cn
Baowei Zhang
zhangbw@ahu.edu.cn

† These authors have contributed equally to this work

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Species are the cornerstone in many domains of biology research, which make accurate species delimitation 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. The phylogenetic results showed that six specific clusters existed in the *H. chinensis* group. Bayesian Phylogenetics and Phylogeography (BPP) analysis indicated that six distinct species exist due to the high probability values ($>$0.95), which were also supported by the Bayes factor (BF) analysis. The divergence time of the *H. chinensis* group was estimated to date back to 18.84 million years ago (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 Mountain, and Taiwan. In right-about colonization, it was 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 may be closely related to the progressive uplift of the Qinghai-Tibetan Plateau (QTP) and historical climate change. Our study provided evidence for species delimitation and speciation process within the *H. chinensis* group. Our study supported the hypothesis that the evolutionary divergence in this species group was a consequence of the progressive uplift of the QTP and environmental change.

Keywords: ***Hyla chinensis*** **group, species delimitation, phylogeny, biogeography, evolutionary history**

INTRODUCTION

For biogeography, abiotic factors (e.g., climate changes and tectonic events) and biological factors (e.g., interspecific or intraspecific interactions, competition, and predation) act as the major drivers temporally and geographically for biological evolution and diversification (Benton, 2009). 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 patterns, such as the relatively small populations with well-defined geographical boundaries (Shepard and Burbrink, 2011; Huang et al., 2017; Pan et al., 2019). In southern China, many mountains (e.g., Hengduan Mountains, Qinling Mountains, Daba Mountains, Wuyi Mountains, and Dabie Mountains) are scattered, which form potential spatially isolated sky islands, providing various microhabitats with beneficial conditions for the speciation process of endemic species (Gao et al., 2015; Zhen et al., 2016). 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 (Päckert et al., 2012; Favre et al., 2015).

Species are considered the cornerstone of research in biology fields (e.g., evolutionary biology, biogeography) (Aldhebiani, 2018), which makes appropriate and accurate species delimitation increasingly meaningful (Yang and Rannala, 2010; Grummer et al., 2014; Blair and Bryson, 2017; Kajtoch et al., 2017; Kotsakiozi et al., 2018; Sheridan and Stuart, 2018). The genus *Hyla* (Hylidae, Anura) comprised 35 recent described species (19 species distributed in Eurasia; 16 species distributed in North and Central America) (Frost, 2014; Li et al., 2015). *Hyla chinensis* group, mainly distributed in China, is one of the species complexes in *Hyla*. As for the number of species identified in the *H. chinensis* group, it is controversial (Hua et al., 2009; Li et al., 2015). One supported that it included seven species (*Hyla annectans*, *H. chinensis*, *Hyla hallowelli*, *Hyla sanchiangensis*, *Hyla simplex*, *Hyla tsinlingensis*, and *Hyla zhaopingensis*) (Hua et al., 2009); the other study supported only six 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*) (Li et al., 2015). Combining 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 (Smith et al., 2005; Wiens et al., 2006), which may be inferred that the speciation of *H. chinensis* group may be from northern China to southern China. However, the phylogenetic tree in the study by Li et al. (2015) disclosed that the base clades of *H. chinensis* group were all located in southern China, which may be a hint of another expansion route of the *H. chinensis* group.

Using genetic data and multiple analysis methods to solve taxonomic uncertainties enables us to disclose phylogenetic topology and speciation process. Here, we reveal a phylogeny of the *H. chinensis* group based on multiple mitochondrial and nuclear genes covering currently described species or subspecies within the *H. chinensis* group (Li et al., 2015). 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.

MATERIALS AND METHODS

Ethics Statement

In this study, the sample collection of *H. tsinlingensis* and *H. chinensis* was conducted by a long-term investigation project on amphibian diversity in Dabie Mountains and Huangshan Mountain. This investigation project and sample collection were approved by the 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 (Li et al., 2015) and chose two species (*Hyla arborea*, *Hyla orientalis)* as outgroups. Additionally, our own specimens (17 *H. tsinlingensis* individuals and two *H. chinensis* individuals) were collected from Dabie Mountains and Huangshan Mountain during 2011 to 2014, all samples were non-invasive sampling and the specimens were stored in School of Life Sciences, Anhui University, China (**Figure 1**). Details on specimen vouchers and GenBank accession numbers and specimen sites are listed in **Table 1**.

Laboratory Methods

The proteinase K digestion and phenol/chloroform extraction method were used to extract total genomic DNA (Sambrook et al., 1989). For combined previous sequence data (Li et al., 2015), the same genes were selected based on published primers and new primers (**Supplementary Table S1**), 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) (Wiens et al., 2005).

All PCRs were performed within the same conditions in 30 μl volume: 10–40 ng of genomic DNA, 15 μl 2 × EasyTaq PCR SuperMix polymerase (containing 1 U Ex Taq, 0.4 mM dNTP, 3 mM Mg^{2+}; TransGen Biotech) and 0.2 μM of primers. PCRs 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 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 semiautomated sequencer (PE Applied Biosystems).

Sequence Processing and Phylogenetic Analyses

The DNA analysis package DNASTAR Lasergene Seqman and EditSeq v.7.1 were used to proofread or assemble the resulting

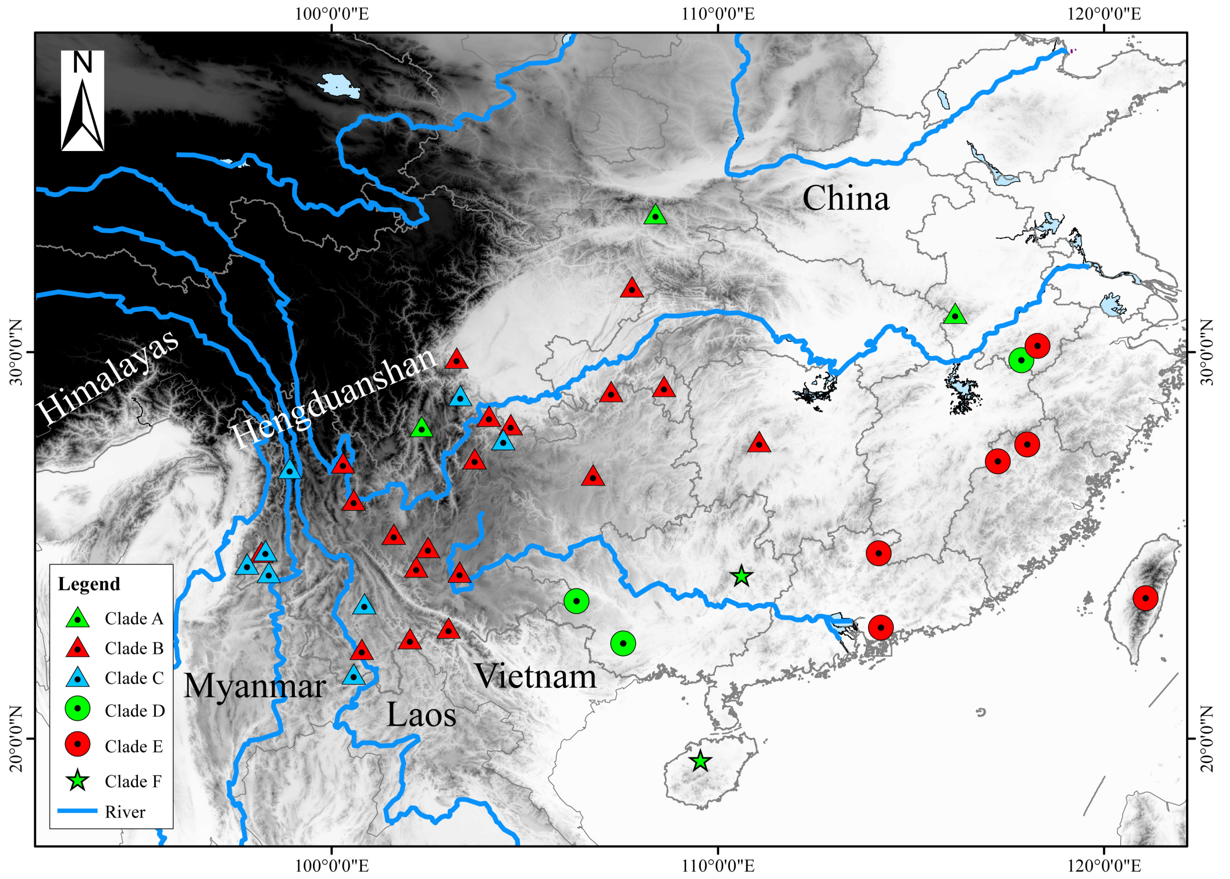

FIGURE 1 | Map with the locality samples of the *Hyla chinensis* group in this study. The sampling sites of each clade **(A–F)** were marked with different triangles or dots in different colors. These clades **(A–F)** correspond to the clades in **Figures 2**, **3**. The black and white coloration represents elevation.

sequences of all genes (Burland, 1999) with default parameters, and the nucleotide sequences were checked by eyes. All the genes were concatenated for analysis and aligned in MEGA v.7.0 (Tamura et al., 2011). Aligned sequences had a total length of 2,474 bp (12S rRNA, 815 bp; 16S+tRNA+ND1, 1,172 bp; 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 conduct species tree, Bayes factor (BF) delimitation (BFD) analyses (Sullivan and Joyce, 2005), 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 conduct the phylogenetic reconstruction [maximum likelihood (ML); Bayesian] and Bayesian Phylogenetics and Phylogeography (BPP) analysis (Rannala and Yang, 2003; Yang and Rannala, 2010).

Before phylogenetic analysis, the software jModeltest v.2 (Darriba et al., 2012) was used to find the best-fit nucleotide substitution model of each gene using Bayesian information criterion (BIC), and this optimal model (GTR+G, 12S; GTR+I+G, 16S+tRNA+ND1+POMC) was selected and implemented in all downstream analyses. 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 (Ronquist and Huelsenbeck, 2003). 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 ML tree was generated with RAxML v.7.0.3 (Stamatakis, 2008) using the GTR model for mitochondrial and nuclear datasets. Support of nodes was calculated with 1,000 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 with 1,000 bootstrap replicates by Kimura two-parameter model on mitochondrial genes in MEGA v.7.0 (Tamura et al., 2011).

Divergence Time Estimation

Mitochondrial genes were used to estimate divergence times among *H. chinensis* group in BEAST v.1.8.0 (Drummond et al., 2012). An MCMC approach with uncorrelated lognormal relaxed molecular clock for rate variation was set. Two independent runs were performed, consisting of 10 million generations, each run sampling every 1,000 generations with a burn-in set to 10% of

TABLE 1 | Samples, with sampling site, museum voucher nos., and GenBank accession nos. of corresponding sequences.

Taxon	Locality	Specimen voucher no./ isolate no.	GenBank No. (MtDNA: 12S, 16S, tRNA-Leu and ND1; NuDNA: POMC)					Distribution areas	Source
			12S	**16S**	**tRNA-Leu**	**ND1**	**POMC**		
Hylidae									
Hylinae									
Hyla									
Hyla annectans	China: Binchuan, Yunnan	KIZDL100502	KP742535	KP742664	KP742664	KP742664	–	Y	Li et al., 2015
Hyla annectans	China: Binchuan, Yunnan	KIZBC100601	KP742552	KP742681	KP742681	KP742681	–	Y	Li et al., 2015
Hyla annectans	China: Yulong, Yunnan	KIZLJ100602	KP742540	KP742669	KP742669	KP742669	–	Y	Li et al., 2015
Hyla annectans	China: Fugong, Yunnan	CAS215021	AY819421	DQ055813	DQ055813	DQ055813	DQ055786	Y	Li et al., 2015
Hyla annectans	China: Weixi, Yunnan	KIZ200905320	KP742522	KP742651	KP742651	KP742651	–	Y	Li et al., 2015
Hyla annectans	China: Lvchun, Yunnan	KIZ200905639	KP742523	KP742652	KP742652	KP742652	–	Y	Li et al., 2015
Hyla annectans	China: Yuanyang, Yunnan	KIZ200905641	KP742524	KP742653	KP742653	KP742653	–	Y	Li et al., 2015
Hyla annectans	China: Shuifu, Yunnan	KIZ270806034	KP742525	KP742654	KP742654	KP742654	–	Y	Li et al., 2015
Hyla annectans	China: Shuifu, Yunnan	KIZ270806035	KP742526	KP742655	KP742655	KP742655	–	Y	Li et al., 2015
Hyla annectans	China: Yuexi, Sichuan	KIZLZY20090172	KP742527	KP742656	KP742656	KP742656	–	Y	Li et al., 2015
Hyla annectans	China: Yongde, Yunnan	KIZ200701067	KP742528	KP742657	KP742657	KP742657	–	Y	Li et al., 2015
Hyla annectans	China: Jinggu, Yunnan	KIZJG125	KP742529	KP742658	KP742658	KP742658	–	Y	Li et al., 2015
Hyla annectans	China: Tengchong, Yunnan	KIZGLGS3481	KP742530	KP742659	KP742659	KP742659	KP742498	Y	Li et al., 2015
Hyla annectans	China: Tengchong, Yunnan	KIZGLGS3729	KP742531	KP742660	KP742660	KP742660	KP742499	Y	Li et al., 2015
Hyla annectans	China: Shiwuli, Yunnan	KIZGLGS2658	KP742532	KP742661	KP742661	KP742661	KP742500	Y	Li et al., 2015
Hyla annectans	China: Hongya, Sichuan	CIBLJT070504	KP742533	KP742662	KP742662	KP742662	KP742491	Y	Li et al., 2015
Hyla annectans	China: Jinping, Yunnan	KIZ060821f	KP742534	KP742663	KP742663	KP742663	–	Y	Li et al., 2015
Hyla annectans	China: Tengchong, Yunnan	KIZTC100501	KP742536	KP742665	KP742665	KP742665	–	Y	Li et al., 2015
Hyla annectans	China: Sangzhi, Hunan	KIZSZ100602	KP742537	KP742666	KP742666	KP742666	–	Y	Li et al., 2015
Hyla annectans	China: Sangzhi, Hunan	KIZSZ100603	KP742538	KP742667	KP742667	KP742667	–	Y	Li et al., 2015
Hyla annectans	China: Yunlong, Yunnan	KIZDL100504	KP742539	KP742668	KP742668	KP742668	–	Y	Li et al., 2015
Hyla annectans	China: Menglian, Yunnan	KIZML100602	KP742541	KP742670	KP742670	KP742670	–	Y	Li et al., 2015
Hyla annectans	China: Menghai, Yunnan	KIZMH100601	KP742542	KP742671	KP742671	KP742671	–	Y	Li et al., 2015
Hyla annectans	China: Menghai, Yunnan	KIZMH100602	KP742543	KP742672	KP742672	KP742672	–	Y	Li et al., 2015
Hyla annectans	China: Kunming, Yunnan	KIZKM060501	KP742544	KP742673	KP742673	KP742673	–	Y	Li et al., 2015
Hyla annectans	China: Mile, Yunnan	KIZMLE080502	KP742545	KP742674	KP742674	KP742674	–	Y	Li et al., 2015
Hyla annectans	China: Yuexi, Sichuan	CIBLJT101186	KP742546	KP742675	KP742675	KP742675	KP742494	Y	Li et al., 2015
Hyla annectans	China: Yuexi, Sichuan	CIBJT101187	KP742547	KP742676	KP742676	KP742676	KP742495	Y	Li et al., 2015
Hyla annectans	China: Zhaotong, Yunnan	KIZSK100801	KP742548	KP742677	KP742677	KP742677	–	Y	Li et al., 2015
Hyla annectans	China: Zhaotong, Yunnan	KIZMLZ100801	KP742549	KP742678	KP742678	KP742678	–	Y	Li et al., 2015
Hyla annectans	China: Lvfeng, Yunnan	KIZCX101001	KP742550	KP742679	KP742679	KP742679	–	Y	Li et al., 2015
Hyla annectans	China: Ningliang, Yunnan	KIZNL100901	KP742551	KP742680	KP742680	KP742680	–	Y	Li et al., 2015
Hyla annectans	China: Youyang, Chongqing	CIB20120440	KP742553	KP742682	KP742682	KP742682	KP742489	Y	Li et al., 2015
Hyla annectans	China: Nanchuan, Chongqing	CIBZYC776	KP742554	KP742683	KP742683	KP742683	KP742490	Y	Li et al., 2015
Hyla annectans	China: Nanchuan, Chongqing	CIBZYC777	KP742555	KP742684	KP742684	KP742684	–	Y	Li et al., 2015
Hyla annectans	China: Nanchuan, Chongqing	CIBGP263	KP742556	KP742685	KP742685	KP742685	–	Y	Li et al., 2015
Hyla annectans	China: Nanchuan, Chongqing	CIBGP273	KP742557	KP742686	KP742686	KP742686	–	Y	Li et al., 2015
Hyla annectans	China: Nanchuan, Chongqing	CIBGP274	KP742558	KP742687	KP742687	KP742687	–	Y	Li et al., 2015
Hyla annectans	China: Xuanhan, Sichuan	CIB201105129	KP742559	KP742688	KP742688	KP742688	–	Y	Li et al., 2015
Hyla annectans	China: Xuanhan, Sichuan	CIB201105130	KP742560	KP742689	KP742689	KP742689	–	Y	Li et al., 2015
Hyla annectans	China: Xuanhan, Sichuan	CIB201105131	KP742561	KP742690	KP742690	KP742690	–	Y	Li et al., 2015
Hyla annectans	China: Xuanhan, Sichuan	CIB201105132	KP742562	KP742691	KP742691	KP742691	–	Y	Li et al., 2015
Hyla annectans	China: Xuanhan, Sichuan	CIB201105133	KP742563	KP742692	KP742692	KP742692	–	Y	Li et al., 2015
Hyla annectans	China: Lijiang, Yunnan	CIB3LW0019	KP742564	KP742693	KP742693	KP742693	KP742493	Y	Li et al., 2015
Hyla annectans	China: Yuexi, Sichuan	SCUM060486L	KP742565	KP742694	KP742694	KP742694	–	Y	Li et al., 2015
Hyla annectans	China: Yuexi, Sichuan	SCUM060487L	KP742566	KP742695	KP742695	KP742695	–	Y	Li et al., 2015
Hyla annectans	China: Junlian, Sichuan	CIBGP298	KP742567	KP742696	KP742696	KP742696	–	Y	Li et al., 2015
Hyla annectans	China: Junlian, Sichuan	CIBGP299	KP742568	KP742697	KP742697	KP742697	KP742492	Y	Li et al., 2015
Hyla annectans	China: Guizhou	CIBGZ080537	KP742569	KP742698	KP742698	KP742698	–	Y	Li et al., 2015
Hyla annectans	China: Guizhou	CIBGZ080538	KP742570	KP742699	KP742699	KP742699	–	Y	Li et al., 2015
Hyla chinensis	China: Taiwan	HL1	–	DQ055817	DQ055817	DQ055817	DQ055789	W	Li et al., 2015
Hyla chinensis	China: Zhaowu, Fujian	IOZCAS4796	KP742571	KP742700	KP742700	KP742700	KP742503	W	Li et al., 2015
Hyla chinensis	China: Longmen, Guangdong	CIB200905260	KP742572	KP742701	KP742701	KP742701	–	W	Li et al., 2015

(Continued)

TABLE 1 | Continued

Taxon	Locality	Specimen voucher no./ isolate no.	GenBank No. (MtDNA: 12S, 16S, tRNA-Leu and ND1; NuDNA: POMC)					Distribution areas	Source
			12S	16S	tRNA-Leu	ND1	POMC		
Hyla chinensis	China: Wuyihshan, Fujian	WYS100602	KP742573	KP742702	KP742702	KP742702	KP742502	W	Li et al., 2015
Hyla chinensis	China: Chebaling, Guangdong	CIB20120429	KP742574	KP742703	KP742703	KP742703	–	W	Li et al., 2015
Hyla chinensis	China: Chebaling, Guangdong	CIB20120430	KP742575	KP742704	KP742704	KP742704	KP742505	W	Li et al., 2015
Hyla chinensis	China: Chebaling, Guangdong	CIB20120431	KP742576	KP742705	KP742705	KP742705	–	W	Li et al., 2015
Hyla chinensis	China: Chebaling, Guangdong	CIB20120432	KP742577	KP742706	KP742706	KP742706	–	W	Li et al., 2015
Hyla chinensis	China: Chebaling, Guangdong	CIB20120433	KP742578	KP742707	KP742707	KP742707		W	Li et al., 2015
Hyla chinensis	China: Chebaling, Guangdong	CIB20120434	KP742579	KP742708	KP742708	KP742708	–	W	Li et al., 2015
Hyla chinensis	China: Nanling, Guangdong	CIBGP400	KP742580	KP742709	KP742709	KP742709	KP742504	W	Li et al., 2015
Hyla chinensis	China: Nanling, Guangdong	CIBGP409	KP742581	KP742710	KP742710	KP742710	–	W	Li et al., 2015
Hyla chinensis	China: Tanjiaqiao, Anhui	AHU20140801	MK880293				MK883719	W	This study
Hyla chinensis	China: Tanjiaqiao, Anhui	AHU20140802	MK880294				MK883720	W	This study
Hyla sanchiangensis	China: Xiuning, Anhui	CIB20120435	KP742636	KP742755	KP742755	KP742755	KP742506	W	Li et al., 2015
Hyla sanchiangensis	China: Xiuning, Anhui	CIB20120436	KP742637	KP742756	KP742756	KP742756	–	W	Li et al., 2015
Hyla sanchiangensis	China: Xiuning, Anhui	CIB20120437	KP742638	KP742757	KP742757	KP742757	–	W	Li et al., 2015
Hyla sanchiangensis	China: Xiuning, Anhui	CIB20120438	KP742639	KP742758	KP742758	KP742758	–	W	Li et al., 2015
Hyla sanchiangensis	China: Xiuning, Anhui	CIB20120439	KP742640	KP742759	KP742759	KP742759	–	W	Li et al., 2015
Hyla sanchiangensis	China: Chongzuo, Guangxi	CIBGP1936	KP742641	KP742760	KP742760	KP742760	–	S	Li et al., 2015
Hyla sanchiangensis	China: Nonggang, Guangxi	–	KP742761	KP742761	KP742761	KP742507	–	S	Li et al., 2015
Hyla tsinlingensis	China: Ningshan, Shaanxi	CIBLJT070511	KP742645	KP742764	KP742764	KP742764	KP742496	Z	Li et al., 2015
Hyla tsinlingensis	China: Ningshan, Shaanxi	CIBLJT070512	KP742646	KP742765	KP742765	KP742765	KP742497	Z	Li et al., 2015
Hyla tsinlingensis	China: Ningshan, Shaanxi	SCUM06060005	GQ374901	GQ374905	GQ374905	GQ374905	GQ374917	Z	Li et al., 2015
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140601	MK862405	MK876185	MK876185	MK876185	MK876202	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140602	MK862406	MK876186	MK876186	MK876186	MK876203	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140603	MK862407	MK876187	MK876187	MK876187	MK876204	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140604	MK862408	MK876188	MK876188	MK876188	MK876205	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140605	MK862409	MK876189	MK876189	MK876189	MK876206	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140606	MK862410	MK876190	MK876190	MK876190	MK876207	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140607	MK862411	MK876191	MK876191	MK876191	MK876208	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140608	MK862412	MK876192	MK876192	MK876192	MK876209	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140609	MK862413	MK876193	MK876193	MK876193	MK876210	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140610	MK862414	MK876194	MK876194	MK876194	MK876211	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140611	MK862415	MK876195	MK876195	MK876195	MK876212	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140612	MK862416	MK876196	MK876196	MK876196	MK876213	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140613	MK862417	MK876197	MK876197	MK876197	MK876214	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140614	MK862418	MK876198	MK876198	MK876198	MK876215	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140615	MK862419	MK876199	MK876199	MK876199	MK876216	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140616	MK862420	MK876200	MK876200	MK876200	MK876217	Z	This study
Hyla tsinlingensis	China: Yaoluoping, Anhui	AHU20140617	MK862421	MK876201	MK876201	MK876201	MK876218	Z	This study
Hyla zhaopingensis	China: Zhaoping, Guangxi	CIBZP100601	KP742647	KP742766	KP742766	KP742766	–	N	Li et al., 2015
Hyla zhaopingensis	China: Zhaoping, Guangxi	CIBZP100602	KP742648	KP742767	KP742767	KP742767	–	N	Li et al., 2015
Hyla zhaopingensis	China: Danzhou, Hainan	CIBGP641	KP742649	KP742768	KP742768	KP742768	–	N	Li et al., 2015
Hyla zhaopingensis	China: Danzhou, Hainan	CIBGP642	KP742650	KP742769	KP742769	KP742769	KP742508	N	Li et al., 2015
Outgroup									
Hylidae									
Hylinae									
Hyla									
Hyla arborea	Croatia: Split−Dalmatia County, Kamesnica, Mountain, pool in Donja Korita village	–	DQ055835	DQ055814	DQ055814	DQ055814	DQ055787		
Hyla orientalis	Russia	CIB20120455	KP742630	KP742751	KP742751	KP742751	KP742509		

*"–" represents no molecular data. The distribution areas were consistent with that within **Figure 3**.*

the samples. Tracer v.1.6 was used to check the stationarity of results (Rambaut and Drummond, 2007). TreeAnnotator v.1.8.0 (Rambaut and Drummond, 2007) and FigTree v. 1.4.2 (Rambaut, 2014) was used to annotate and visualize tree information. In the absence of appropriate fossils, we selected several calibration points information from previous work (Li et al., 2015), assuming a normal distribution for the divergence time between *H. arborea* group and the *H. chinensis* group, with a mean of 23 million years ago (Mya) and standard deviation of 3.04 (18–28 Mya).

Bayes Factor Delimitation

The BF is a common species delimitation selection tool in phylogenetics (Sullivan and Joyce, 2005) based on the marginal-likelihood estimates (MLE) *via* path-sampling (PS) and stepping-stone sampling (SS) analyses (Fan et al., 2011; Xie et al., 2011; Li and Drummond, 2012). 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 (Drummond et al., 2012). Coupled with the former studies (Hua et al., 2009; Li et al., 2015) and the above phylogenetic analysis 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 (Heled and Drummond, 2010). 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, totaling 10^8 generations with a chain length of 10^6 generations for 100 path steps.

Bayesian Phylogenetics and Phylogeography

In contrast to the results of our BFD method and 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 (Rannala and Yang, 2003; Yang and Rannala, 2010). This method utilizes a reversible jump MCMC (rjMCMC) algorithm to calculate the posterior probabilities to speciation events that contain more or less lineages (Yang and Rannala, 2010). Between all BPP analyses, probability values ≥ 0.95 were considered a strong support in favor of a speciation event (Leaché and Fujita, 2010). The guide tree was generated from the species tree.

The priors of ancestral population size (θ) and root age (τ) are directly related to the posterior probabilities of each result for the 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 (Leaché and Fujita, 2010; Yang and Rannala, 2010). We evaluated three schemes of the prior of the θ and τ: (1) $\theta = G$ (1:2,000) and $\tau = G$ (1:10), (2) $\theta = G$ (1:2,000) and $\tau = G$ (1:100), (3) $\theta = G$ (2:2,000) and $\tau = G$ (1:10). The parameters of the rjMCMC analyses were set as 500,000 generations with sampling every 50 steps and 100,000 burn-in steps.

Species Tree Inference

The coalescent-based method implemented in *BEAST was used to construct the species tree (Heled and Drummond, 2010) based on mitochondrial genes. Two independent runs of 20 million generations 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 an uncorrected p-distances phylogenetic network with heterozygous ambiguities averaged and normalized by Splitstree v. 4.13.1 (Huson and Bryant, 2006). 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 (Tang and Zhang, 1984; Li and Yang, 1985; Fei et al., 2009; Frost, 2014). The five areas were as follows: 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 (**Figures 1**, **3**). LAGRANGE is a deep-time biogeographical model-based method that allows the incorporation of paleogeographical data (Ree and Smith, 2008; Chacón and Renner, 2014). Taking the effect of the LAGRANGE model components into account, we designed experiments that transform the adjacency matrix, hence, resulting in a total of three experiments (M0, M1, and M2). This is according to the assumption that the *H. chinensis* group, like all organisms, has a lower possibility to disperse over non-adjacent areas than adjacent areas. For this reason, no ranges were forbidden for M0; WZ, SZ, and NZ were forbidden for M1; WZ, NZ, SZ, and NY were forbidden for M2. To select the optimal model, we compared their log-likelihood (the data presented by LAGRANGE); meanwhile, we used the standard cutoff value of two log-likelihood units as indicating a conspicuous imbalance between models, with the less negative likelihood being preferred (Chacón and Renner, 2014). Ancestral areas were reconstructed by dispersal-extinction-cladogenesis model (Garzione et al., 2000) as carried out in the software Lagrange v.20130526 (Ree and Smith, 2008), 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–F) within the *H. chinensis* group (**Figures 2**, **3**). Clade A corresponds to *H. tsinlingensis* and *H. annectans chuanxiensis*, which are mainly located in the Qinling-Dabie mountains; Clade B included *H. annectans*, *H. a. wulingensis*,

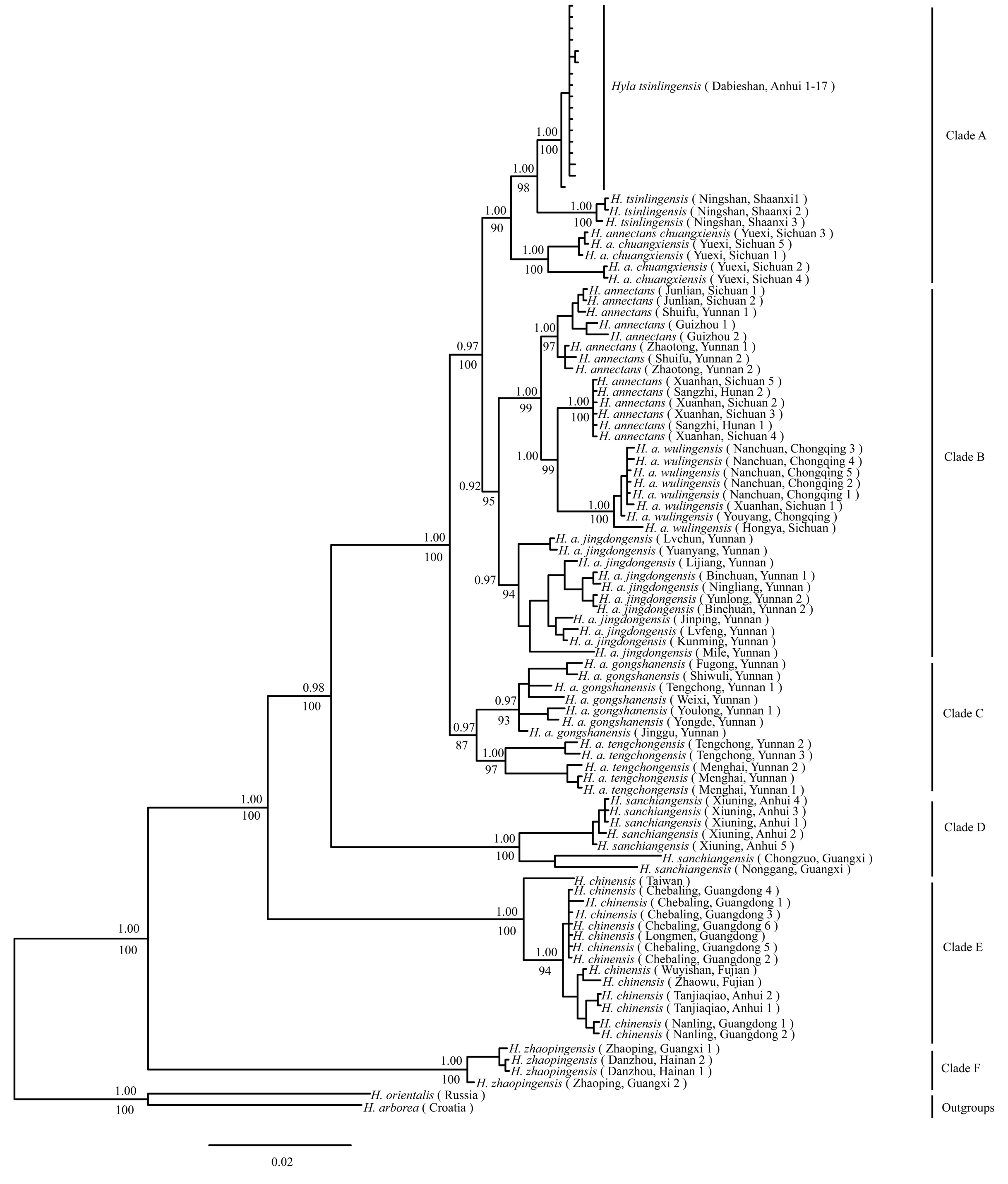

FIGURE 2 | Phylogenetic relationship of the *Hyla chinensis* group by Bayesian phylogenetic and maximum likelihood (ML) analysis based on concatenated sequences (mtDNA and nuclear genes). Values above branches indicate Bayesian posterior probabilities, below bootstrap values of the ML analysis. **(A–F)** represents the six clades within the *H. chinensis* group.

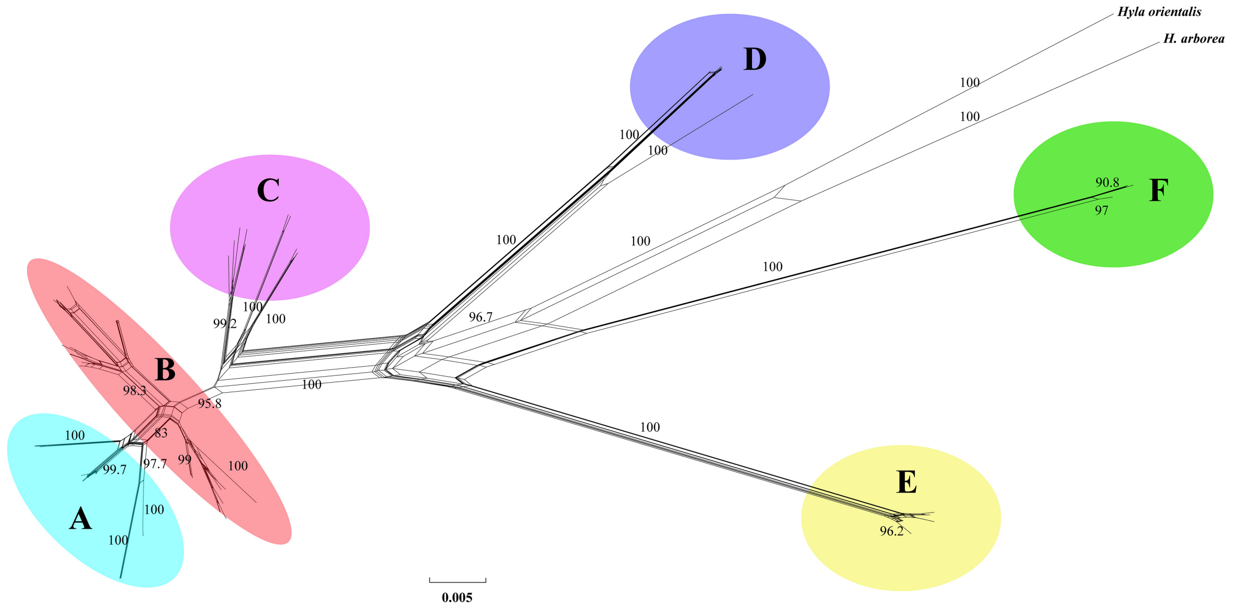

FIGURE 3 | Network constructed from the mtDNA genes of the *Hyla chinensis* group based on uncorrected p-distances using SPLITSTREE. The values on nodes indicate bootstrap support (only values above 75% are shown). **(A–F)** represents the six clades within the *H. chinensis* group.

and *H. a. jingdongensis*, while *H. a. gongshanensis* and *H. a. tengchongensis* are 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*) are located in the Guangxi province, Hainan province, and the Eastern China, respectively (**Figures 2**, **3**). The phylogenetic network of the *H. chinensis* group showed the consistent groupings compared with the phylogenetic methods (**Figure 4**). 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 the *H. chinensis* group was taken place from late Miocene (11.88 Mya) to the early Pleistocene (around 4.82 Mya) (**Figure 3**).

The BFD based on PS (BF, 12.62) and SS (BF, 20.84) models decisively supported six species in the *H. chinensis* group (**Table 2**), corresponding to the six clades disclosed by the phylogenetic tree (**Figures 2**, **3**). BPP methods also supported six separated species due to higher than 0.95 probability values (**Table 3**). Species tree, consistent with BPP tree topology, recovered a concordant, robust phylogenetic topology (**Figure 5**). Pairwise sequence divergence (p uncorrected distance) between hidden species in the *H. chinensis* group ranged from 2.1% (A vs. B) to 11.4% (E vs. F) (**Table 4**).

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 was restricted (**Table 5**). The analyses supported the Southern China (Guangxi-Hainan provinces, Area N) and Eastern China (Area W) as the ancestral area of the *H. chinensis* group, and most speciation events were attributed to the progressive uplift of the Himalayas (**Figure 3** and **Supplementary Table S2**). Additionally, *H. tsinlingensis* 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., Clades A–F) (**Figures 2–4**). Based on BF and BPP methods, the species delimitation suggested that these six genetically distinct clades could be regarded as hidden separated species in the *H. chinensis* group, which also got the support from genetic distance (**Table 4**). In brief, clade A corresponds to *H. tsinlingensis* and *H. annectans chuanxiensis*; clade B included *H. annectans*, *H. a. wulingensis*, and *H. a. jingdongensis*; while *H. a. gongshanensis* and *H. a. tengchongensis* are within clade C. The remaining clades (D–F) corresponded to three putative species (*H. sanchiangensis*, *H. chinensis*, and *H. zhaopingensis*), respectively (**Figures 2**, **3**), which were supported by the various species delimitation analyses. Overall, compared with the study of Li et al. (2015), some minor differences exist: *H. annectans chuanxiensis* belongs to *H. tsinlingensis*, not *H. annectans*; two subspecies of *H. annectans* (*H. a. gongshanensis* and *H. a. tengchongensis*) were regarded as separated species. As for the species not involved (*H. simplex*), we cannot give corresponding conclusions due to missing key samples in our research. As mentioned by previous researchers, further clarification of the relationship of *H. simplex* and *H. zhaopingensis* can be resolved only based on the *H. simplex* from its type locality in Vietnam and in-depth analysis (Li et al., 2015).

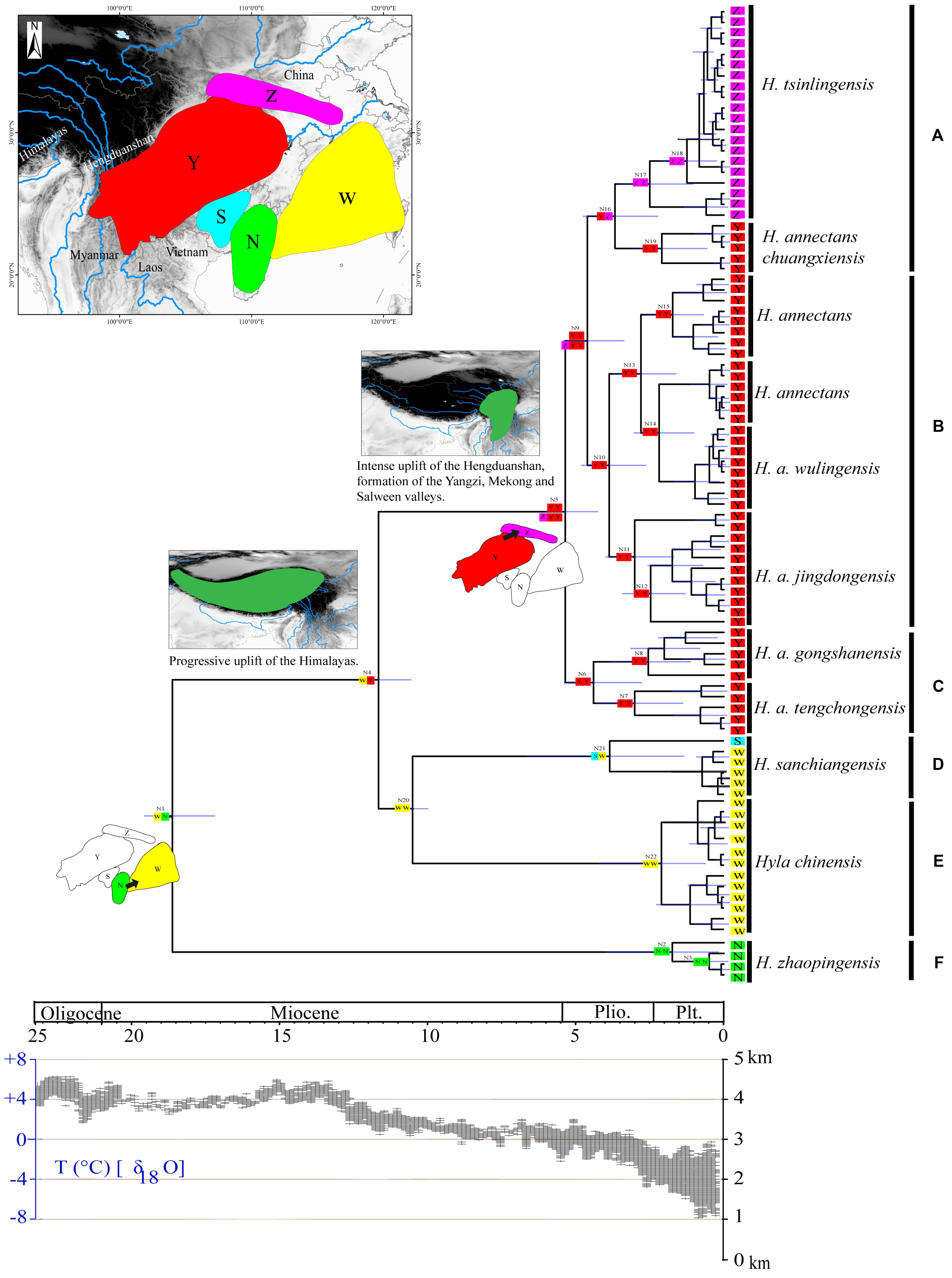

FIGURE 4 | Chronogram and ancestral reconstructions of the *Hyla chinensis* group. **Top panel:** time-calibrated phylogeny of the *H. chinensis* group based on mitochondrial dataset [the light blue bars through the nodes indicate 95% highest posterior densities (HPDs)] and ancestral area reconstruction 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 the *H. chinensis* group including a graphical representation of the extent of uplift of the Tibetan Plateau (TP) through time green shades indicate the portion of the Qinghai-Tibetan Plateau that had achieved altitudes comparable to present day (adapted from Favre et al., 2015). 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 (Zachos et al., 2001, 2008). **(A–F)** represents the six clades within the *H. chinensis* group.

TABLE 2 | Species delimitation results of the *Hyla 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	–	–

MLE, marginal likelihood estimate; BF, Bayes factor; PS, path sampling; SS, stepping stone.

TABLE 3 | The species delimitation results of the *Hyla chinensis* group based on mtDNA and nuclear gene data in Bayesian Phylogenetics and Phylogeography (BPP).

Scheme	Prior distribution		Posterior probabilities
	Θ	τ	
Scheme 1	G (1, 100)	G (1, 2,000)	0.98393
Scheme 2	G (1, 10)	G (1, 2,000)	0.98877
Scheme 3	G (1, 10)	G (2, 2,000)	0.98607

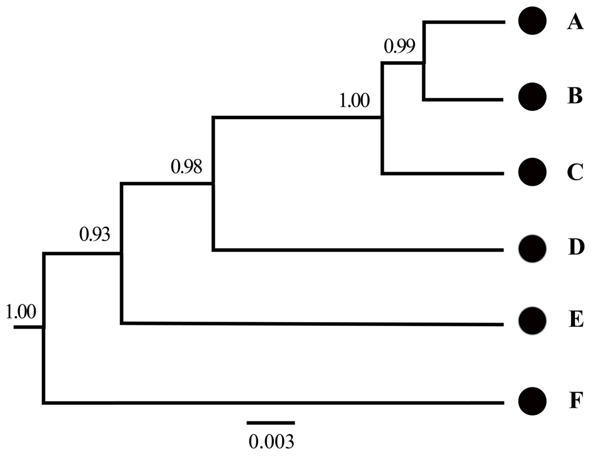

FIGURE 5 | Species tree estimated using BEAST based on mtDNA genes in the *Hyla chinensis* group. The letters **(A–F)** correspond to the clades in **Figures 2**, **3**. The values on nodes are Bayesian posterior probabilities.

TABLE 4 | Average genetic distances by Kimura two−parameter model among six candidate species within the *Hyla chinensis* group based on mtDNA genes.

Clade	A	B	C	D	E	F
A						
B	0.010*					
C	0.015*	0.015*				
D	0.066*	0.063*	0.056*			
E	0.082*	0.073*	0.071*	0.090*		
F	0.100*	0.096*	0.096*	0.105*	0.110*	

*Significant tests are indicated with an asterisk (*P < 0.01).*

TABLE 5 | Comparison of different dispersal models in Lagrange.

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

M0, unconstrained; M1, dispersal from southern China to the Qinling−Dabie mountains were restricted from W, N, S to Z; M2, dispersal from southern China to the Qinling−Dabie mountains and to the eastern of the Tibetan Plateau were restricted from W, N, S to Z and from N to Y. N, the 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.

In addition, for the distribution pattern of the clade D, this clade includes samples from Anhui and Guangxi; it seems that a large geographic gap exists between these places, which may hint the sampling incompleteness within this clade (Fei et al., 2012). These results represent a definitive molecular evidence for the taxonomic revision of each clade within the *H. chinensis* group. Nevertheless, in order to revise the group from a comprehensive perspective, a thorough quantitative multivariate analysis of the diagnostic morphological features and more key samples of unexplored species (e.g., *H. simplex*) is still required, and ideally, additional ecological evidence is required. During the Middle Eocene to Early Oligocene (45–34 Mya), the *Hyla* originated from North America, then diffused to China through the Bering Land Bridge, thus forming a branch of *Hyla* (Smith et al., 2005; Wiens et al., 2006; Li et al., 2015). In this study, the dated phylogenetic tree indicated that the Clade F (about 18.84 Mya) is at the base of the *H. chinensis* group and contains *H. zhaopingensis* in Southern China (Guangxi province). Six putative hidden species in the *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 Clades D and E) in the *H. chinensis* group occurs in Southern and Eastern in China during the Middle Miocene (ca. 18–10 Ma). *Hyla* is a small, arboreal, and semiaquatic frog; prefers to live in warm and damp environments, which widely inhabits boscages, paddy fields, or edges of rivers; and breeds in still water in ponds or paddy fields (Fei, 2005). During this period, the southern China humid climate is conducive to the survival of the species. For example, paleobotanical data indicated that the southeastern of the QTP has a warm and humid climate, was dominated by subtropical vegetation during the Miocene (Jacques et al., 2011), which had provided an opportunity for the first stage of speciation in the *H. chinensis* group.

The second stage of speciation in the *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 margins (mostly province of Yunnan, Sichuan, and Qinghai) led to the formation of some rivers; the Hengduanshan hot spot of biodiversity was composed of those areas (Favre et al., 2015). 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 (Kutzbach et al., 1993; Tang et al., 2013). The East Asian Monsoon system has controlled China's climate at that time, and this condition brought moisture air from the ocean to East China (Liu et al., 2009). Combining the geological events, they may be contributed to the second stage of speciation in the *H. chinensis* group.

In conclusion, although a previous study had demonstrated that the *Hyla* originated from North America, then diffused to China *via* Beringia during the Middle Eocene to Early Oligocene (Smith et al., 2005; Wiens et al., 2006; Li et al., 2015), it did not mean that the speciation of the *H. chinensis* group may be from northern China to southern China. Under the framework of speciation time within the *H. chinensis* group, the rapid uplifting mountain ranges (the Tibetan Plateau and its adjacent mountain) formed a blocky orographic barrier for many endemic species (Favre et al., 2015), which also played an important role in the formation of the Asian monsoon system (Guo et al., 2008; Song et al., 2010; Tang et al., 2013). Additionally, three East Asian monsoon intensification periods (∼15 Ma, ∼8 Ma, and 4–3 Ma) (Wan et al., 2007; Molnar et al., 2010; Jacques et al., 2011) also had urged the formation of humid and warm climates in south China (Sun and Wang, 2005), which was favorable for geographical dispersal, especially for amphibians (Che et al., 2010; Wu et al., 2013; Yan et al., 2013; Ye et al., 2013). 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 (Che et al., 2010; Wu et al., 2013; Yan et al., 2013; Ye et al., 2013; Favre et al., 2015). Therefore, based on those results, unlike the history of the *Hyla* evolution (North America→Beringia→China) (Smith et al., 2005; Wiens et al., 2006; Li et al., 2015), we can infer 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 the *H. chinensis* group was full of competing. Until now, no research focuses 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 Clades A to 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, diagnostic morphological characters and other ecological evidences are still needed to supply for providing the integrative revision of this species group.

DATA AVAILABILITY STATEMENT

The accession number(s) can be found in the table within this article.

ETHICS STATEMENT

The animal study was reviewed and approved by the Anhui Normal University.

AUTHOR CONTRIBUTIONS

BZ, JL, and XW conceived the study. PY and TP contributed to sample collection. PY, GW, TP, XK, IA, and WZ carried out the laboratory work. PY, GW, TP, and XK analyzed the data and wrote the manuscript with contributions from BZ, XW, JL, PY, and TP. IA corrected the language. All authors approved the final version of the manuscript and agreed to be held accountable for its content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00234/full#supplementary-material

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