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A REVISED PHYLOGENY OF HOLARCTIC TREEFROGS (GENUS *HYLA*) BASED ON NUCLEAR AND MITOCHONDRIAL DNA SEQUENCES

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ABSTRACT: The treefrog genus *Hyla* (Anura: Hylidae) consists of at least 31 species found in North America, Central America, Europe, and Asia and is the only genus of hylids that occurs outside the New World. Despite intensive work on the phylogeny of the genus in the past few years, several problems still exist regarding relationships within *Hyla*. These problems include the unusual placements of *H. gratiosa* and *H. walkeri* in some recent studies and the relatively limited taxon sampling of Asian species. In the present study, we revisit the phylogeny of *Hyla* to address some of these problems. First, we tested the unexpected placements of *H. gratiosa* and *H. walkeri* by sampling additional individuals of these species. Our results show that the unusual placements of *H. gratiosa* and *H. walkeri* in previous studies were most likely due to a mislabelled tissue sample and a misidentified specimen, respectively. Second, we included two species of Asian *Hyla* not included in previous phylogenies. Our study provides additional evidence for two separate colonizations of *Hyla* from the New World into Asia, and suggests an unusual biogeographic pattern in the Asian *Hyla* clades.

Key words: Asia; Biogeography; Eurasia; *Hyla*; North America; Phylogeny; Systematics

HYLIDAE is the second largest family of amphibians, with at least 852 species and 49 genera currently recognized (AmphibiaWeb, 2008). Recent revisionary studies (Faivovich et al., 2005; Wiens et al., 2005) have reclassified hylid frogs into three subfamilies: Hylinae, Pelodryadinae, and Phyllomedusinae. Within Hylinae, the formerly speciose and polyphyletic genus *Hyla* was dismantled and redefined by Faivovich et al. (2005) as a smaller, monophyletic group of North American, Central American and Eurasian hylids. *Hyla* is the only genus of hylids that is found outside the New World, with 5 species in Europe, 10 species in Asia, and the other 16 species in North and Central America (8 in the U.S., 2 shared by the US and Mexico, and 6 in Mexico and Guatemala; IUCN, 2009).

The genus *Hyla* offers an interesting system in which to study the ecological and evolutionary causes and consequences of patterns of historical biogeography and species richness. Previous studies have focused on many related topics, such as key innovations that allow the invasion of tropical hylids into temperate regions (Hedges, 1986; Walton, 1993); mechanisms for reproductive isolation that explain the extensive sympatry of North American hylids (Gerhardt, 1994; Oldham and Gerhardt, 1975); effects of ecological factors, including predation and habitat variability, on species richness and species distributions (Leips et al., 2000; Resetarits and Wilbur, 1989); and niche evolution and conservatism associated with parallel patterns of species richness in different lineages and continents (Smith et al., 2005).

Early studies based on morphological and biochemical data suggested that all three genera of Holarctic hylids (*Acris*, *Pseudacris*, and *Hyla*) arose from a single invasion of South American hylids into North America (Anderson, 1991). The Holarctic hylids were thought to have expanded northward through California, eastward to eastern North America, and westward across Beringia into Eurasia. Hylids are then thought to have undergone in-situ speciation in each region,

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producing assemblages of endemic species in western North America, eastern North America, and Eurasia. In addition, several groups within *Hyla* were suggested by analyses based on morphological and biochemical data (Anderson, 1991), including the *Hyla eximia* group (*H. arenicolor*, *H.euphorbiacea*, *H. eximia*, *H. plicata,* and *H. walkeri*) and *Pseudacris regilla* group (*P. cadaverina* and *P. regilla*) in western North America (with the *H. eximia* group extending into Central America), the *Hyla cinerea* group (*H. cinerea*, *H. gratiosa,* and *H. squirella*), *Hyla versicolor* group (*H. avivoca*, *H. chrysoscelis,* and *H. versicolor*), and *Pseudacris crucifer* group (*P. crucifer*) in eastern North America, and the *Hyla arborea* group (all Asian and European species) in Eurasia (Table I in Anderson, 1991). However, the composition of some groups was uncertain, the placements of *H. femoralis* and *H. andersonii* were unclear, and the monophyly of the *H. arborea* group was in doubt.

Several recent phylogenetic studies based on DNA sequence data (Faivovich et al., 2005; Lemmon et al., 2007; Smith et al., 2005, 2007; Wiens et al., 2005) have converged on a somewhat different picture of Holarctic hylid phylogeny. These analyses showed that there were two separate invasions of North America from Central America: the *Acris-Pseudacris* clade and the genus *Hyla*. Formerly a large and polyphyletic genus, the genus *Hyla* was redefined as a monophyletic group of Holarctic and Central American species by Faivovich et al. (2005), and this grouping has been supported in subsequent studies (Smith et al., 2005, 2007; Wiens et al., 2005, 2006). However, the *H. arborea* group was found to be polyphyletic, with *H. japonica* nested within a paraphyletic *H. eximia* group. This phylogenetic pattern was interpreted as a second invasion of Asian *Hyla* from the New World (Faivovich et al., 2005; Smith et al., 2005; Wiens et al., 2005).

Despite these insights into Holarctic hylid phylogeny, several ambiguities still exist regarding relationships within *Hyla*. First, *H. gratiosa* was placed as the sister taxon of *H. cinerea* by both Faivovich et al. (2005) and Lemmon et al. (2007), rather than nested within the *H. versicolor* group (i.e., with *H. andersonii*, *H. avivoca*, *H. chrysocelis*, *H. femoralis*, and *H. versicolor*) as found by Smith et al. (2005, 2007). Second, Faivovich et al. (2005) suggested that the Asian species *H. japonica* (*H. arborea* group) was the sister taxon to *H. walkeri* (*H. eximia* group) from Mexico and Guatemala. However, that result was based on a specimen of *H. walkeri* taken from the pet trade for which no locality data are available. *Hyla walkeri* was not sampled by Smith et al. (2005, 2007) or Wiens et al. (2006), but this taxon was included in the phylogenies of Smith et al. (2007) and Wiens et al. (2006) based on the data from Faivovich et al. (2005). Third, taxon sampling of species outside of North America is still relatively weak. For example, only 3 of the 10 Asian species have been sampled so far.

In the present study, we revisit the phylogeny of *Hyla* species and attempt to address these problems. First, we test the unusual placements of *H. gratiosa* and *H. walkeri* by sampling additional individuals of these species. Second, we add two more species of Asian *Hyla* not included in previous phylogenies. We sequenced four genes for each species (two mitochondrial, two nuclear) and added these data to the 10-gene dataset used in Smith et al. (2007; which included data from the previous studies) to estimate the phylogenetic relationships within *Hyla*.

Materials and Methods

Taxon sampling for the present study includes all the species of *Hyla* sampled in Smith et al. (2007). Furthermore, we sequenced an additional individual of *H. gratiosa* (DSM 5; Daniel S. Moen field series; currently being accessioned at the American Museum of Natural History, AMNH A181719) from Ocala National Forest, Marion County, Florida, USA (latitude N29°16.147′, longitude W81°42.822′). This specimen has the color pattern characteristic of this species (i.e., many dark rounded spots, unique among eastern North American *Hyla*; Conant and Collins, 1998). Thus we confidently identified the specimen as *H. gratiosa*.

Similarly, an additional individual of *H. walkeri* (JJW 2260; John J. Wiens field series, being accessioned in the Museo de Zoología, Facultad de Ciencias, Universidad Nacional

TABLE 1.—Primers used for amplification and sequencing.

	Primer sequence (listed 5′ to 3′)	Source
12S		
t-Phe-frog	ATAGCRCTGAARAYGCTRAGATG	Modified "MVZ 59" Graybeal (1997)
t-Val-frog	TGTAAGCGARAGGCTTTKGTTAAGCT	Wiens et al. (2005)
ND1		
16S-frog	TTACCCTRGGGATAACAGCGCAA	Wiens et al. (2005)
ND1-R1	TCCTCCCTATCAAGGAGGTCC	Smith et al. (2005)
c-myc exon 2		
c-myc 1U	GAGGACATCTGGAARAARTT	Crawford (2003)
c-myc ex2d R	TCATTCAATGGGTAAGGGAAGACGACC	Wiens et al. (2005)
c-myc exon 3		
c-myc ex3F2	AYGTNCCYATYCAYCAGCACAACT	Wiens et al. (2005)
c-myc ex3R3	TCKCGNAKGAGYCKYCGCTCRTC	Wiens et al. (2005)
c-myc 3L	GTCTTCCTCTTGTCRTTCTCYTC	Wiens et al. (2005)
c-myc ex3F	CCCACCAGTCCAGACCTCACCACAG	Wiens et al. (2005)
POMC		
POMC-1	GAATGTATYAAAGMMTGCAAGATGGWCCT	Wiens et al. (2005)
POMC-2	TAYTGRCCCTTYTTGTGGGCRTT	Wiens et al. (2005)
POMC-6	TCTGCMGAGTCACCRGTGTTTC	Smith et al. (2005)
POMC-7	TGGCATTTTTGAAAAGAGTCAT	Smith et al. (2005)

Autónoma de México) from the city of San Cristóbal de las Casas, state of Chiapas, Mexico, was confidently identified and sequenced. This specimen was assigned to *H. eximia* based on morphological criteria (from Duellman, 1970). Furthermore, this is the only species of *Hyla* that occurs in the state of Chiapas, and this species was previously recorded for this locality (Duellman, 1970).

We obtained and sequenced two Chinese species that were not included in previous studies, *H. immaculata* (SCUM 06060001; from the district of Shashi within the city of Jingzhou, Hubei Province, China) and *H. tsinlingensis* (SCUM 06060005; from the town of Jiangkou, Ningshan County, Shanxi Province, China). Tissue samples of these species were provided by Sichuan University Museum (SCUM).

The sister group to *Hyla* is a clade including the genera *Isthmohyla, Smilisca*, and *Tlalocohyla* (Smith et al., 2005, 2007; Wiens et al., 2006). We included two species from each genus using data from Smith et al. (2007) and used them as outgroup taxa.

For each new sample, we sequenced two mitochondrial genes (ribosomal small subunit [12S; 986 base pairs], and NADH dehydrogenase subunit 1 with up to 342 bp of the adjacent tRNA genes [ND1; 1191 bp total]) and two nuclear genes (proopiomelanocortin A gene [POMC; 523 bp], and portions of exons 2 [430 bp] and 3 [404 bp] of the v-myc myelocytomatosis viral oncogene homolog [*c-myc*]) as in Smith et al. (2005). We extracted DNA from ethanol-preserved tissues using proteinase K digestion (DNeasy tissue kit from Qiagen) and amplified it using PCR with primer sequences listed in Table 1. We purified both strands of each PCR product and sequenced them using an ABI 3100 automated sequencer.

We added new sequences for each gene to the existing data for each gene from Smith et al. (2007). We first analyzed each gene separately to assess the placement of each new individual and to test for possible contamination or other lab errors, and then combined new sequences into the 10-gene dataset used in Smith et al. (2007). In addition to 12S, ND1, POMC, and *c-myc*, this data set includes two additional mitochondrial genes: cytochrome *b* (cytb; 385 bp) and ribosomal large subunit (16S; 1493 bp); and four nuclear genes: recombination activating gene 1 (RAG1; 428 bp), tyrosinase (TYR; 530 bp), seven in absentia homolog 1 (SIAH1; 397 bp) and rhodopsin (RHO; 316 bp). These additional genes were sequenced by Faivovich et al. (2005) for most of the same species. These were combined with the data of Smith et al. (2007), although this involved concatenating

data from different individuals for different genes to represent each species. For most species, this concatenation was unambiguous, given that Faivovich et al. (2005) and Smith et al. (2005) generated very similar phylogenies for these taxa. However, we did not concatenate data from different individuals for *Hyla gratiosa* or *Hyla walkeri*, but treated these individuals as separate units in the analysis. We included a total of 169 gene sequences for 22 *Hyla* species in the phylogenetic analyses. GenBank numbers and original sources are shown in Table 2. In theory, we could have sequenced these six additional genes for the four added taxa, but the four genes we sequenced were adequate to place these taxa in the phylogeny, especially in view of the consistent placements of these taxa in the phylogenies of individual genes (Fig. 1).

Sequences for additional Eurasian *Hyla* species are available in GenBank. These include 12S (GenBank number AF218709, unpublished sequence by Suh, J. H., February 2000) and cytb (GenBank number AF205096, Lee et al., 1999) sequences for *H. suweonensis*, cytb sequences for *H. sarda* (GenBank number AY960652, unpublished sequence by Kluetsch, C. F. C., Nasher, A. K., Misof, B., Naumann, C. M., and Grosse, W. R., December 2008) and *H. intermedia* (Canestrelli et al., 2007), and *c-myc* exon 2 sequence for *H. simplex* (GenBank number DQ055775, Smith et al., 2005). However, since there are only fragmentary data for these species (average of 432 aligned nucleotide positions for each of these four species compared to >2,000 aligned positions for each species included in the former 10-gene dataset), we did not include these species in the individual gene analyses, but did an additional combined gene analysis based on a new 10-gene dataset that includes these Eurasian *Hyla* species. We performed this additional analysis using Bayesian and likelihood methods (see below) to test if these additional species are still placed in the clades that are established by the analysis of the 10-gene dataset without these species.

We aligned sequences using Clustal X. 1.83 (Thompson et al., 1994). Instead of merely fitting new sequences to the existing alignment from Smith et al. (2007), we realigned all the sequences following Wiens et al. (2005) by comparing alignment results under different gap opening penalties (12.5, 15, 17.5). Gene regions that differ in alignment results with different gap opening penalties were considered ambiguously aligned and were excluded from phylogenetic analyses. However, in this study, no ambiguous regions were found.

We analyzed data using Bayesian and likelihood methods. We analyzed each individual gene separately to look for clades that are incongruent and strongly supported by each dataset, possibly indicating different phylogenetic histories of each gene (after Wiens, 1998). Similarly, we analyzed combined nuclear genes and mitochondrial genes separately, to look for strongly supported incongruence between these datasets. A clade was considered strongly supported if it had a likelihood bootstrap value $\geq$ 70% (see Felsenstein, 2004; Hillis and Bull, 1993) or a Bayesian posterior probability $\geq$ 0.95 (Alfaro et al., 2003; Erixon et al., 2003; Huelsenbeck and Rannala, 2004). We found no strongly supported incongruence between genes or between the combined nuclear and combined mitochondrial genes (Fig. 1). We therefore combined all the data to increase the overall sample size of characters.

We implemented Bayesian analyses in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). The best substitution model for each gene was selected using the Akaike information criterion in MrModeltest version 2.0 (Nylander, 2004). We evaluated the best partitioning strategy within and between genes (e.g., different partitions for different genes and different codon positions within genes) using comparison of Bayes factors (Brandley et al., 2005; Nylander et al., 2004), based on comparing the harmonic mean of the log-likelihoods of the post-burn-in trees from analyses with and without partitions within each gene. These analyses showed that the best combination of models and partitioning strategies was GTR+I for c-myc and GTR+I+Γ for the other genes (general time reversible model, with additional parameters for invariant sites [I] and variation in rates among variable sites [Γ]). These analyses also showed strong support for using separate partitions for each gene and the following partitions within genes: 12S (stems, loops), ND1 (structural

TABLE 2.—GenBank accession numbers for DNA sequences analyzed in this study. [a] = sequences taken from Wiens et al. (2005), [b] = Faivovich et al. (2005), [c] = Darst and Cannatella (2004), [d] = Moriarty and Cannatella (2004), [e] = Smith et al. (2005), [f] = Smith et al. (2007), [g] = unpublished sequence from GenBank (Jiang and Zhou, October 2000). * = Data for *Hyla gratiosa* or *H. walkeri* from Faivovich et al. (2005). ** = Data for *H. gratiosa* from Smith et al. (2005).

	Mitochondrial genes			
	12S	16S	Cytb	ND1
H. andersonii	AY291115[c]	AY843598[b]	AY843819[b]	DQ055812[e]
H. annectans	AY819421[a]	AY843600[b]	AY843821[b]	DQ055813[e]
H. arborea	DQ055835[e]	AY843601[b]	AY843822[b]	DQ055814[e]
H. arenicolor	AY819363[a]	AY843603[b]	AY843824[b]	AY819494[a]
H. avivoca	DQ055836[e]	AY843605[b]	AY843828[b]	DQ055815[e]
H. chinensis	AF315129[g]	—	—	DQ055817[e]
H. chrysocelis	AY291116[d]	AY291116[d]	—	—
H. cinerea	AY819366[a]	AY549327[b]	AY843846[b]	AY819498[a]
H. euphorbiacae	DQ055837[e]	AY843625[b]	AY843855[b]	DQ055818[e]
H. eximia	AY291113[d]	AY843626[b]	AY843856[b]	—
H. femoralis	DQ055838[e]	AY843627[b]	AY843859[b]	DQ055819[e]
H. gratiosa	GQ374899	—	—	GQ374903
*H. gratiosa**	DQ055839[f]	—	—	DQ055820[f]
*H. gratiosa***	—	AY843630[b]	AY843862[b]	—
H. immaculata	GQ374900	—	—	GQ374904
H. japonica	DQ055840[e]	AY843633[b]	AY843866[b]	DQ055821[e]
H. meridionalis	AY819370[a]	—	—	AY819502[a]
H. plicata	DQ055842[e]	—	—	DQ055826[e]
H. savignyi	DQ055843[e]	AY843665[b]	AY843907[b]	DQ055829[e]
H. squirella	AY819378[a]	AY843678[b]	AY843923[b]	AY819510[a]
H. tsinlingensis	GQ374901	—	—	GQ374905
H. versicolor	AY819441[a]	AY843682[b]	AY843928[b]	DQ055831[e]
H. walkeri	GQ374902	—	—	GQ374906
*H. walkeri**	AY843684[b]	AY843684[b]	AY843930[b]	—
H. wrightorum	AY819368[a]	—	—	AY819500[a]
Isthmohyla pseudopuma	AY819435[a]	AY843656[b]	AY843897[b]	DQ055827[e]
Isthmohyla zeteki	AY819442[a]	—	—	—
Smilisca baudinii	DQ388698[f]	AY843762[b]	AY844007[b]	DQ388762[f]
Smilisca cyanosticta	AY819393[a]	AY843763[b]	AY844008[b]	AY819525[a]
Tlalocohyla godmani	DQ388689[f]	—	—	DQ388755[f]
Tlalocohyla picta	DQ388693[f]	AY843654[b]	AY843894[b]	DQ388758[f]

	Nuclear genes						
	MYCex2	MYCex3	POMC	RAG1	RHO	SIAH1	TYR
H. andersonii	DQ055756[e]	DQ055730[e]	DQ055785[e]	—	AY844572[b]	AY844798[b]	AY844044[b]
H. annectans	DQ055757[e]	DQ055731[e]	DQ055786[e]	AY844388[b]	AY844574[b]	AY844800[b]	AY844045[b]
H. arborea	DQ055758[e]	DQ055732[e]	DQ055787[e]	AY844389[b]	AY844575[b]	—	AY844046[b]
H. arenicolor	AY819197[a]	AY819271[a]	AY819112[a]	AY844391[b]	AY844577[b]	AY844802[b]	AY844048[b]
H. avivoca	DQ055759[e]	DQ055733[e]	—	AY844394[b]	AY844581[b]	AY844805[b]	AY844051[b]
H. chinensis	DQ055761[e]	—	DQ055789[e]	—	—	—	—
H. chrysocelis	—	—	—	—	—	—	—
H. cinerea	AY819201[a]	AY819275[a]	AY819116[a]	AY844408[b]	AY844597[b]	AY844816[b]	AY844063[b]
H. euphorbiacae	DQ055763[e]	DQ055736[e]	DQ055791[e]	—	AY844606[b]	AY844823[b]	AY844072[b]
H. eximia	—	—	—	—	—	AY844824[b]	AY844073[b]
H. femoralis	DQ055764[e]	DQ055737[e]	DQ055792[e]	—	AY844609[b]	AY844826[b]	AY844074[b]
H. gratiosa	GQ374907	GQ374911	GQ374915	—	—	—	—
*H. gratiosa**	DQ055765[f]	DQ055738[f]	DQ055793[f]	—	—	—	—
*H. gratiosa***	—	—	—	AY844418[b]	AY844611[b]	AY844829[b]	AY844076[b]
H. immaculata	GQ374908	GQ374912	GQ374916	—	—	—	—
H. japonica	DQ055766[e]	DQ055739[e]	DQ055794[e]	AY844420[b]	AY844615[b]	AY844833[b]	AY844078[b]
H. meridionalis	AY819205[a]	AY819279[a]	AY819120[a]	—	—	—	—
H. plicata	DQ055771[e]	DQ055744[e]	—	—	—	—	—
H. savignyi	DQ055774[e]	DQ055747[e]	DQ055801[e]	—	AY844654[b]	—	AY844107[b]
H. squirella	AY819213[a]	AY819287[a]	AY819128[a]	AY844462[b]	AY844670[b]	AY844882[b]	AY844119[b]

TABLE 2.—Continued.

	Nuclear genes						
	MYCex2	MYCex3	POMC	RAG1	RHO	SIAH1	TYR
H. tsinlingensis	GQ374909	GQ374913	GQ374917	—	—	—	—
H. versicolor	DQ055778[e]	DQ055749[e]	DQ055805[e]	AY844465[b]	AY844675[b]	AY844885[b]	AY844124[b]
H. walkeri	GQ374910	GQ374914	GQ374918	—	—	—	—
*H. walkeri**	—	—	—	AY844466[b]	AY844677[b]	—	AY844125[b]
H. wrightorum	AY819203[a]	AY819277[a]	AY819118[a]	—	—	—	—
Isthmohyla pseudopuma	DQ055772[e]	DQ055745[e]	DQ055799[e]	AY844444[b]	AY844643[b]	AY844861[b]	AY844101[b]
Isthmohyla zeteki	DQ055779[e]	DQ055750[e]	DQ055806[e]	—	—	—	—
Smilisca baudinii	DQ388738[f]	DQ388747[f]	DQ388720[f]	—	AY844749[b]	AY844946[b]	—
Smilisca cyanosticta	AY819228[a]	AY819302[a]	AY819143[a]	AY844524[b]	AY844750[b]	AY844947[b]	AY844184[b]
Tlalocohyla godmani	DQ388730[f]	DQ388744[f]	DQ388710[f]	—	—	—	—
Tlalocohyla picta	—	—	DQ388714[f]	AY844442[b]	AY844640[b]	AY844858[b]	AY844099[b]

regions, loops, three codon positions), and three codon positions for all protein-coding genes. For each separate and combined analysis, we ran two replicate searches for 5.0×10^6 generations each, sampling every 1000 generations and using default parameters. The standard deviation of split frequencies was below 0.01 after each run. We assessed stationarity by examining plots of log-likelihood values over time. Trees generated prior to achieving stationarity were discarded as burn-in. We also used the *Sump* command to evaluate whether separate analyses converged on similar mean log-likelihoods. All analyses reached stationarity before 50,000 generations. The phylogeny was estimated as the majority-rule consensus of the pooled post burn-in trees from the two searches.

We carried out maximum likelihood analyses using RAxML version 7.0.3 (Stamatakis, 2006). This implementation of the likelihood method allows for the optimization of individual substitution models for different partitions, and the current version newly employs the GTR+I+ Γ model. Thus, we applied the same combination of models (GTR+I+ Γ) and partitioning strategies between and within genes as in the Bayesian analyses. Two hundred inferences were executed using RAxML on distinct randomized parsimony starting trees with 1,000 nonparametric bootstrap replicates.

RESULTS

The combined analysis of 10 genes included 25 ingroup taxa and 7083 aligned nucleotide positions, of which 2010 were variable and 1341 were parsimony informative. Patterns of variation in the 10 genes are summarized in Table 3. Separate analysis of the combined nuclear and combined mitochondrial genes using both Bayesian and maximum likelihood methods resulted in similar topologies (Fig. 1). Combined analysis of 10 genes using Bayesian and maximum likelihood methods produced the same topology, with most nodes strongly supported (Fig. 2). The additional Bayesian and maximum likelihood analyses that include four additional Eurasian species (for which only fragmentary data are available) produced the same topology and confirmed that the additional species are placed in the clades established in the former analyses (Fig. 3).

Our phylogeny of *Hyla* is similar to that in Smith et al. (2007). The monophyly of *Hyla* was strongly supported in analyses of *c-myc*, the combined nuclear genes, and the concatenated dataset of 10 genes. We identified five major clades, which generally correspond to the species groups summarized in Anderson (1991), with an *H. arborea* group, *H. japonica* group, *H. cinerea* group, *H. versicolor* group, and *H. eximia* group (Fig. 2). The *H. cinerea* group (*H. cinerea*, *H. gratiosa,* and *H. squirella*) was strongly supported as monophyletic in analyses of the nuclear genes *c-myc* and POMC, combined nuclear genes, and the concatenated dataset of 10 genes. Both the data for *H. gratiosa* used in Faivovich et al. (2005) and the data for *H. gratiosa* in the present study (from DSM 5)

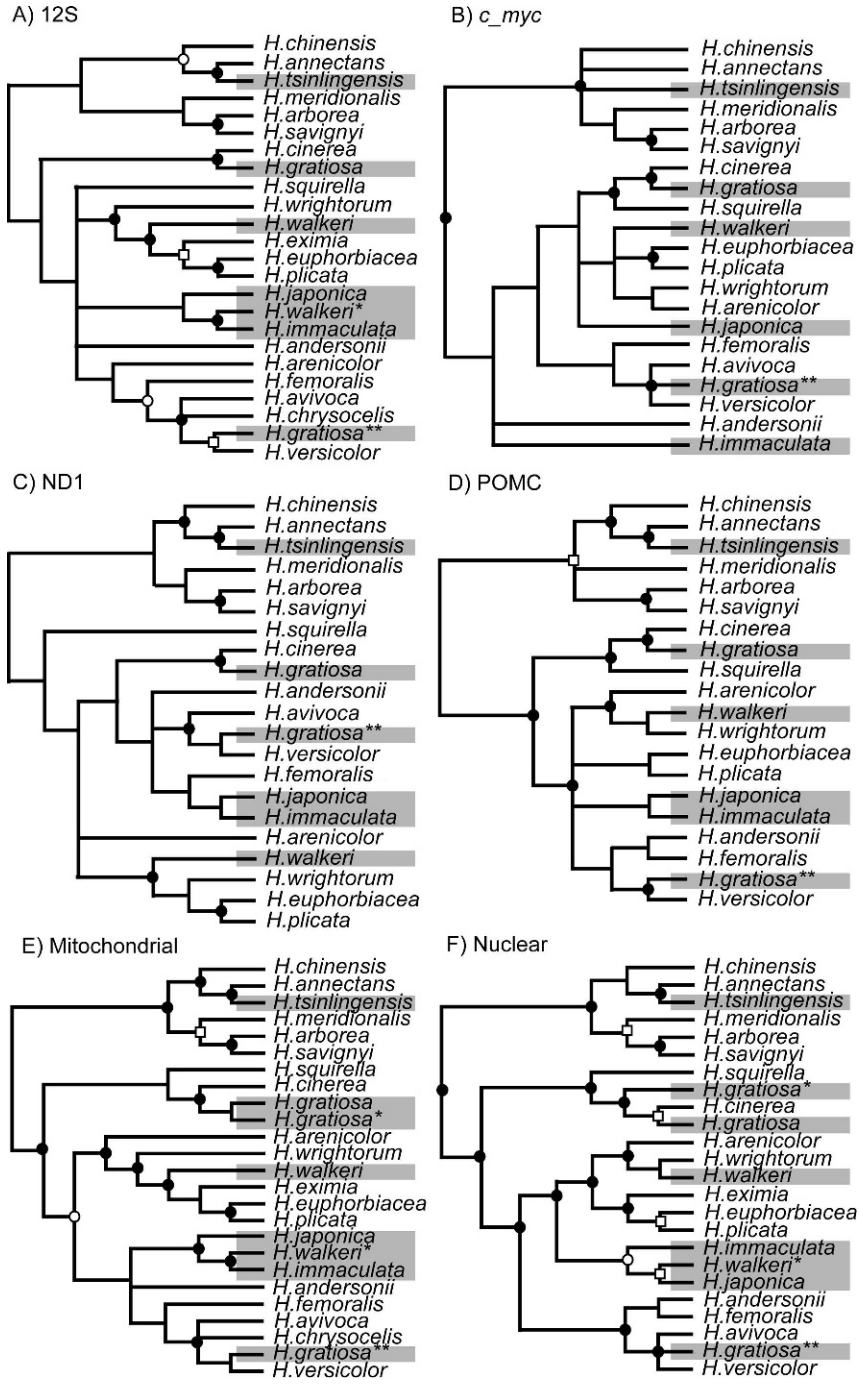

FIG. 1.—Phylogeny of the hylid frog genus *Hyla* based on separate Bayesian and maximum likelihood analyses of (A) 12S (mitochondrial DNA), (B) *c-myc* exons 2 and 3 (nuclear DNA), (C) ND1 (mitochondrial DNA), (D) POMC (nuclear DNA), (E) combined mitochondrial genes, and (F) combined nuclear genes. Filled circles indicate nodes that are strongly supported in both Bayesian and likelihood analyses (bootstrap values $\geq$ 70% and Bayesian posterior probabilities $\geq$ 0. 95). Open circles indicate nodes strongly supported only by Bayesian analysis. Open squares indicate nodes strongly supported only by maximum likelihood. For specimens of questionable identification (from *H. gratiosa* and *H. walkeri*), a single asterisk (*) indicates that the data are from Faivovich et al. (2005), whereas two (**) indicate that the data are from Smith et al. (2005). Outgroup taxa were pruned out after the analysis.

TABLE 3.—Patterns of sequence variation for the genes used in this study.

	Mitochondrial genes				Nuclear genes					
	12S	16S	Cyt b	ND1	*c-myc*	POMC	RAG1	RHO	SIAH1	TYR
Nucleotides	986	1493	385	1191	834	523	428	316	397	530
Variable sites	314	502	153	544	91	153	55	42	37	119
Parsimony-informative sites	214	313	129	425	51	94	18	21	17	59

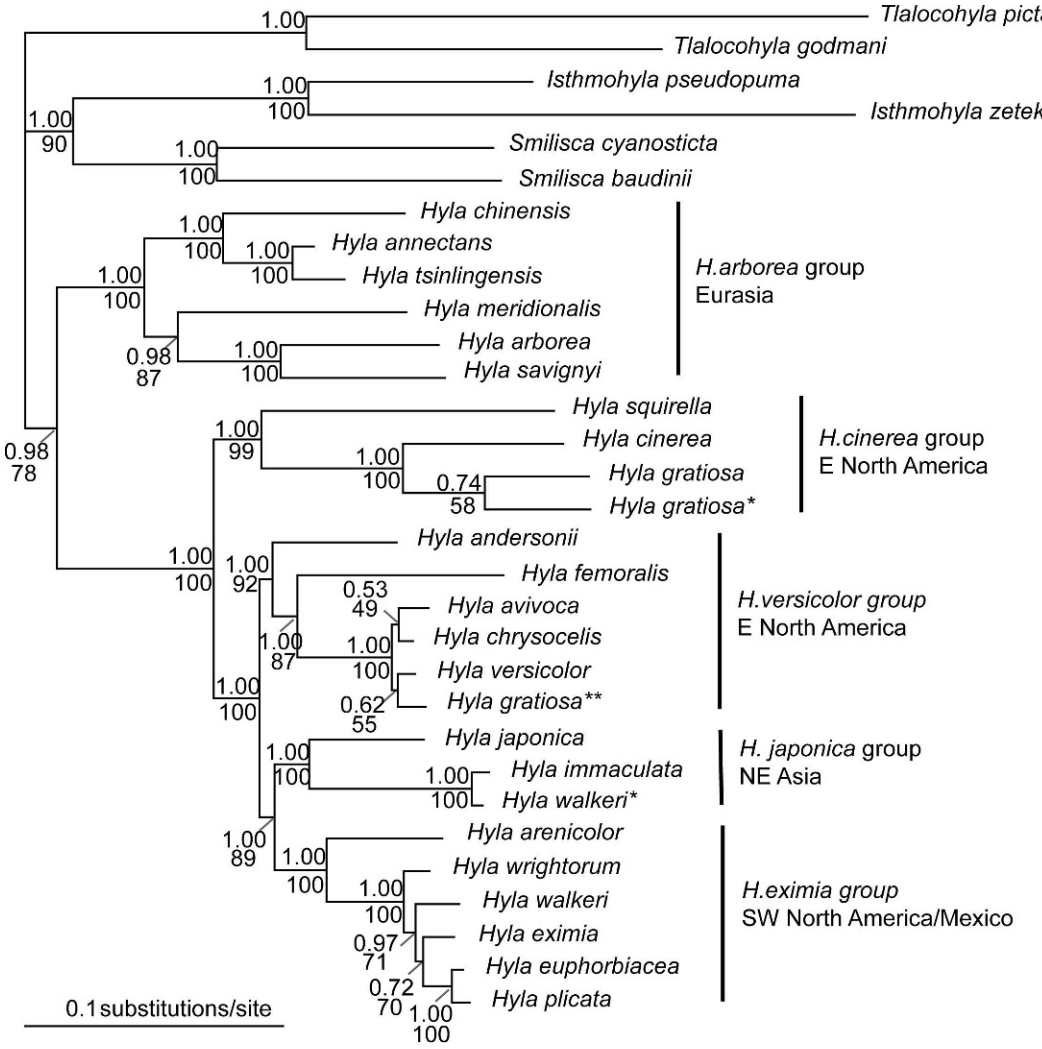

FIG. 2.—Phylogeny of the hylid frog genus *Hyla* based on Bayesian and maximum likelihood analysis of 10 genes combined. Numbers above branches are Bayesian posterior probabilities and numbers below are likelihood bootstrap support values. Group assignment and biogeographic distribution of major clades are listed on the right. For specimens of questionable identification (from *H. gratiosa* and *H. walkeri*), a single asterisk (*) indicates that the data are from Faivovich et al. (2005), whereas two (**) indicate that the data are from Smith et al. (2005).

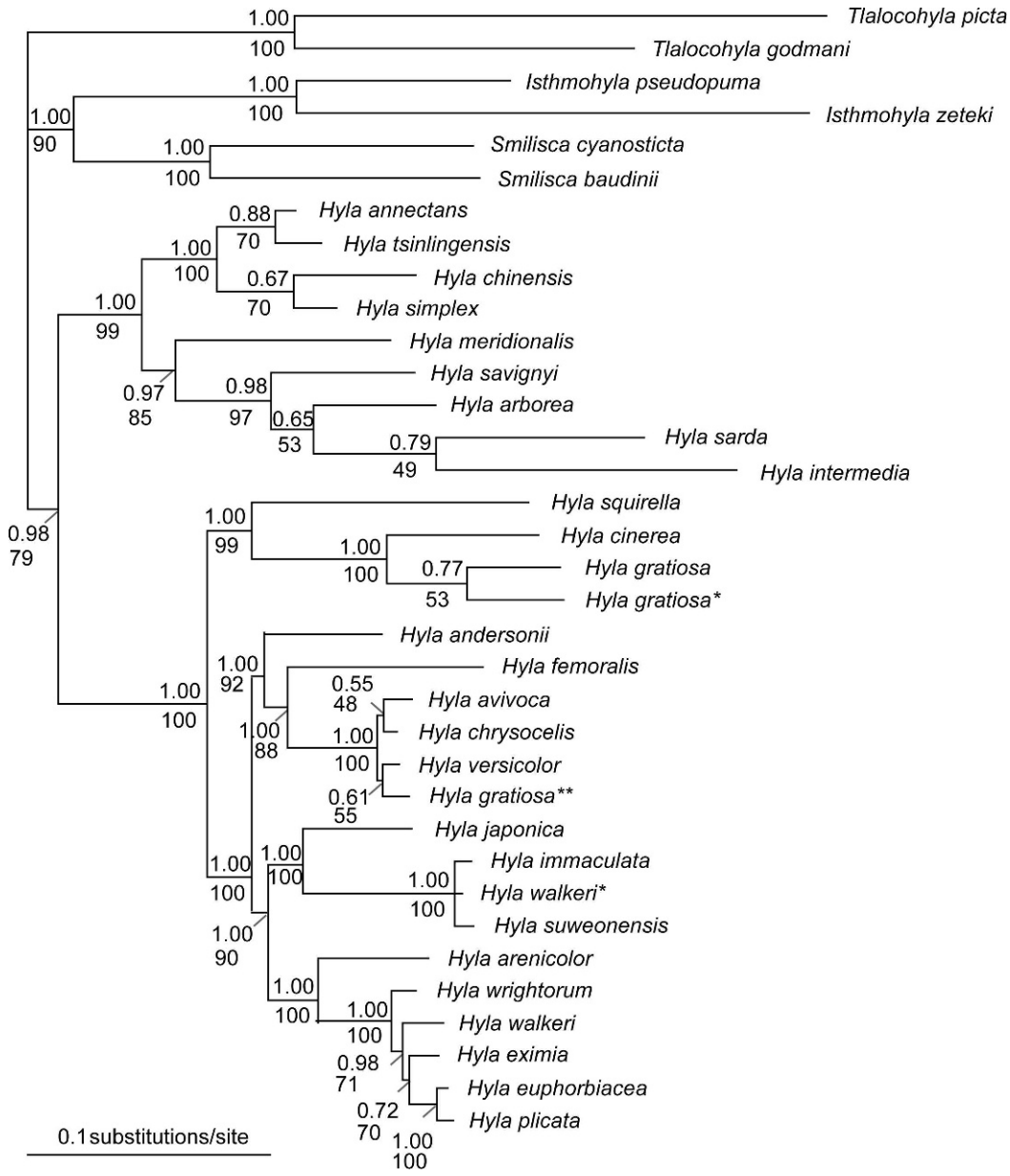

FIG. 3.—Phylogeny of the genus *Hyla* based on Bayesian and maximum likelihood analysis of the combined 10-gene dataset, including four additional Eurasian species for which only fragmentary data are available. Numbers above branches are Bayesian posterior probabilities and numbers below are likelihood bootstrap support values. For specimens of questionable identification (from *H. gratiosa* and *H. walkeri*), a single asterisk (*) indicates that the data are from Faivovich et al. (2005), whereas two (**) indicate that the data are from Smith et al. (2005).

placed *H. gratiosa* as the sister taxon of *H. cinerea*. However, the putative specimen of *H. gratiosa* first used in Smith et al. (2005; see also Smith et al., 2007; Wiens et al., 2006) was placed in the *H. versicolor* group in all the analyses, as found by those authors.

The *H. arborea* group consists of most European and Asian *Hyla*, including the

newly sampled Asian species *H. tsinlingensis*. The monophyly of the *H. arborea* group was strongly supported in all the analyses except for the analyses of 12S and ND1. Among the sampled species, the *H. japonica* group consists of *H. japonica* and *H. immaculata*. The close relationship between the *japonica* group and the *eximia* group was supported in analyses of the combined nuclear genes (Fig. 1) and the concatenated dataset of 10 genes (Fig. 2). Data from the putative specimen of *H. walkeri* from Faivovich et al. (2005) place it within the *H. japonica* group, and show it to be very similar to *Hyla immaculata*. In contrast, the individual of *H. walkeri* sampled in the present study was placed in the *H. eximia* group in all the analyses.

The additional Bayesian and maximum likelihood analyses that include four additional Eurasian species also strongly support the monophyly of the two Eurasian *Hyla* groups and their relationships with other *Hyla* groups in the New World (Fig. 3). The analyses confirm that the Asian *H. simplex* is clustered with *H. chinensis* in the Asian clade of the *H. arborea* group; *H. sarda* and *H. intermedia* are clustered together in the European clade of the *H. arborea* group; the Korean *H. suweonensis* is clustered with *H. immaculata* and the putative specimen of *H. walkeri* from Faivovich et al. (2005) in the *H. japonica* group (Fig. 3). Although the within-clade relationships between the four Eurasian species and other species in their clades are poorly supported, the placements of the four species into its corresponding clade are all strongly supported (Fig. 3). The placement of these species in the expected groups, despite missing ~94% of their characters, supports results from simulations (e.g., Wiens, 2003; Philippe et al., 2004; Wiens and Moen, 2008) and empirical data (e.g., Driskell et al., 2004; Philippe et al., 2004; Wiens et al., 2005) which suggest that highly incomplete taxa can be accurately placed in phylogenetic analyses, especially when the overall number of characters is large.

Discussion

Several recent studies have addressed the phylogeny of *Hyla* (e.g., Faivovich et al., 2005; Smith et al., 2005, 2007; Wiens et al. 2005, 2006), but there have been persistent inconsistencies regarding the placment of *H. gratiosa* and *H. walkeri*, and taxon sampling within the genus is still far from complete. In this study, we have tried to resolve these problems, at least in part.

First, we find that the resampled *Hyla gratiosa* is strongly supported as the sister taxon of *H. cinerea* in our study, a result that is consistent with other phylogenetic studies based on either mitochondrial or combined nuclear and mitochondrial sequence data (Faivovich et al., 2005; Lemmon et al., 2007). Earlier morphological and biochemical studies (reviewed in Anderson, 1991) also suggested the inclusion of *H. gratiosa* in the *H. cinerea* group. In fact, *H. gratiosa* is sometimes confused morphologically and acoustically with *H. cinerea*, and its hybridization with *H. cinerea* has been recorded and widely studied (e.g., Gerhardt et al., 1980; Höbel and Gerhardt, 2003).

In theory, it is possible that the abnormal position of *H. gratiosa* in Smith et al. (2005) occurred because of introgression of one or more genes of a member of the *H. versicolor* group into the genome of some *H. gratiosa* (including the individual they used). However, Smith et al. (2005) analyzed multiple nuclear and mitochondrial genes, and the concordance among individual genes in that study makes introgression seem very unlikely (i.e., a hybrid individual should contain a mixture of genes from each parental species, and different genes should suggest different phylogenetic placements for the individual).

The anomalous position of *H. gratiosa* in the phylogenetic analyses of Smith et al. (2005) is more likely explained by a misidentified voucher specimen or a mislabelled tissue sample. One possibility is that the putative specimen of *H. gratiosa* (LSU 84850) used by Smith et al. (2005) is actually a specimen of the *H. versicolor* group. Using digital images of this specimen provided by C. Austin, we found that this specimen has the diagnostic character of *H. gratiosa* (a profusion of small, dark, rounded spots on the dorsum; Conant and Collins, 1998) and lacks the diagnostic characters shared by the members of the *H. versicolor* group (a light spot below the eye and a gray dorsum with large dark blotches in *H. avivoca, H. chrysocelis,* and *H. versicolor*; Conant and Collins,

1998) to which this putative individual of *H. gratiosa* appeared very closely related. These observations indicate that the voucher specimen itself was not misidentified. However, the tissue number of this specimen (LSUMZ H-19067) is similar to that of a specimen of *H. versicolor* (LSUMZ H-19077), which appears to have been processed at the same time (C. Austin, personal communication). Therefore, we hypothesize that the sequence data for *H. gratiosa* used by Smith et al. (2005) were actually from *H. versicolor*. Similarly, the sequence data for *H. gratiosa* used in Smith et al. (2007) and Wiens et al. (2006) are a mixture of six genes from an actual *H. gratiosa* from Faivovich et al. (2005) and four genes from tissues of *H. versicolor* from Smith et al. (2005).

Second, our results suggest that the anomalous position of *H. walkeri* may be due to a misidentified specimen. The specimen of *H. walkeri* (AMNH-A 168406) used by Faivovich et al. (2005) was actually taken from the pet trade, and no locality data were available. The molecular data from Faivovich et al. (2005) for this specimen were later used by Smith et al. (2005, 2007) and Wiens et al. (2006). Phylogenies based on these data suggest that there is a clade formed by *H. walkeri* and *H. japonica,* which is the sister taxon to the *H. eximia* group. Our study, using a *H. walkeri* specimen of known provenance, supports the monophyly of the traditional *H. eximia* group (including *H. walkeri*). We find that the *eximia* group is the sister taxon to the *H. japonica* group (*H. japonica* and *H. immaculata*). *Hyla walkeri* occurs in far southern Mexico and Guatemala, the southernmost part of the distribution of New World *Hyla*. In contrast, *H. japonica* and *H. immaculata* are distributed in northeastern Asia. Given that we have included almost all New World *Hyla* in our phylogenetic analyses, the clustering of *H. walkeri* with *H. immaculata* seems very unlikely, unless all the species that have geographic distribution between them are extinct. Furthermore, the very short branch length connecting *H. immaculata* and *H. walkeri* makes this biogeographic connection seem even more improbable (in fact, it appears that the putative specimen of *H. walkeri* may actually be conspecific with *H. immaculata*). Our study offers a more reasonable position of *H. walkeri* in terms of its distribution (i.e., it is closely related to other Central American *Hyla*). We hypothesize that the putative specimen of *H. walkeri* used by Faivovich et al. (2005) represents *H. immaculata* or a very closely related species.

We have examined the putative specimen of *H. walkeri* (AMNH-A 168406) used by Faivovich et al. (2005), along with reference specimens of *H. walkeri* (AMNH-A 77630–77634) and *H. immaculata* (AMNH-A 21804, 21806, 21815, 21817; these specimens are catalogued as *H. japonica*, but come from the Chinese mainland in Anhui Province, where *H. immaculata* occurs but *H. japonica* does not; Fei et al., 1999). In fact, *H. walkeri* and *H. immaculata* are quite similar in overall structure, size, and coloration. According to Fei et al. (1999), *H. immaculata* is distinct in lacking the dark brown dorsolateral stripe on the flanks (which begins near the nostril) that occurs in many other *Hyla* species in Asia and elsewhere, including *H. walkeri* (Duellman, 1970). However, the specimens of *H. immaculata* that we examined do have this dark stripe, but it is indistinct, and much fainter than in *H. walkeri*. Further, in *H. immaculata*, there is a white border over above the dark dorsolateral stripe. This white striping also occurs along the outer margins of the front and hind limbs and above the anus. The white striping is present but much less distinct in *H. walkeri*. The putative specimen of *H. walkeri* (AMNH-A 168406) used by Faivovich et al. (2005) agrees more with *H. immaculata* than *H. walkeri*, in having a very faint dark dorsolateral stripe on the flanks and more distinct white stripes bordering the dark dorsolateral stripe and along the margins of the limbs and above the anus. Thus, we think that this specimen more likely represents *H. immaculata* than *H. walkeri*, based on both genetic and morphological criteria.

Third, our results allow placement of two additional species of Asian *Hyla* into the phylogeny (*H. immaculata* and *H. tsinlingensis*). The placement of these taxa supports the idea that there have been two separate invasions of Asia by New World *Hyla*, and that the previously recognized *Hyla arborea* group is paraphyletic (Faivovich et al., 2005; Smith et al., 2005). This idea is further confirmed by the additional analysis that

includes four more Eurasian species (Fig. 3), although these species have only fragmentary data.

Considering our results based on DNA sequence data and chromosomal and immunological results from previous authors, it is now possible to tentatively assign most Asian *Hyla* to one of these two clades. Based on karyological data, Anderson (1991) previously divided Eurasian *Hyla* into two groups. One group (*H. japonica* group) is distributed in far eastern Asia and includes *H. japonica* and *H. suweonensis.* These two species share the presence of an NOR (nuclear organizer region) in chromosome 6, with representatives of the *H. eximia* group and some of the *H. versicolor* group (including *H. avivoca*, *H. chrysoscelis,* and *H. versicolor*). The other species studied by Anderson (1991), including *H. arborea*, *H. chinensis*, *H. hallowelli*, *H. meridionalis*, and *H. savignyi* were placed in a second group (*H. arborea* group). An NOR is found in chromosomes 10 or 11 of these species, as well as in most North American species of *Hyla* (although we acknowledge that this may be plesiomorphy rather than a synapomorphy). Analyses based on immunological data (Riehl et al., 1995) support a close relationship between *H. japonica* and *H. suweonensis*, and between *H. chinensis* and *H. hallowelli*. Using the same fragmentary sequence data from GenBank, Smith et al. (2005) weakly supported that *H. suweonensis* was in the larger clade of *Hyla* that includes *H. japonica*, and our additional analysis significantly supported the placement of *H. suweonensis* in the *H. japonica* group (Fig. 3). Therefore, *H. suweonensis* seems to be a member of the *H. japonica* group. Our additional analysis also strongly supported the placement of *H. simplex* in the Asian clade of *H. arborea* group. In addition, *H. sanchiangensis* shares a NOR in chromosome 9 with *H. annectans*, and shares an additional NOR in chromosome 10 with *H. chinensis* (Li et al., 1991). Therefore, *H. simplex* and *H. sanchiangensis* are likely to be members of the *H. arborea* group. In summary, it seems likely that the Asian clade of the *H. arborea* group includes *H. hallowelli*, *H. sanchiangensis*, and *H. simplex*, in addition to *H. annectans, H. chinensis*, and *H. tsinlingensis* (confirmed by DNA analyses). The *H. japonica* group seems to include *H. immaculata, H. japonica,* and *H. suweonensis*. The assignment of *H. zhaopingensis* remains uncertain, although it strongly resembles some members of the *H. arborea* group (e.g., *H. annectans, H. chinensis, H. sanchiangensis, H. tsinglingensis*) in having dark spots on the flanks and posterior surfaces of the thighs (Fei et al., 2009).

Although the phylogeny of Asian *Hyla* remains incomplete, the existing data suggest some intriguing biogeographic patterns. Except for the Korean species *H. suweonensis* and the Japanese species *H. hallowelli*, all Asian *Hyla* occur in China, including *H. immaculata*, *H. japonica*, *H. chinensis*, and *H. sanchiangensis* in northeastern China, *H. tsinlingensis* in central China, *H. simplex* and *H. zhaopingensis* in mid-southern China (with *H. simplex* also in Vietnam), and *H. annectans* in southwest China (but also extending into Myanmar, India, Thailand, and Vietnam; for range maps of all species see IUCN, 2009). Given the available information on the phylogeny and biogeography of *Hyla*, the two groups of Asian *Hyla* appear to be largely parapatric but with a narrow area of overlap around 30°N latitude (IUCN, 2009). Asisan species of the *H. arborea* group (including *H. annectans*, *H. chinensis*, *H. hallowelli*, *H. sanchiangensis, H. simplex*, and *H. tsinlingensis;* as well as *H. zhaopingensis*) are restricted to the Oriental zoogeographic realm (south of the Tsinling Mountains). In contrast, the *H. japonica* group (*H. immaculata*, *H. japonica,* and *H. suweonensis*) occurs in the Palaearctic realm (northeastern Asia). The Tsinling Mountains have long been recognized as the boundary between the two biogeographic realms (Zhang, 1979). Hoffmann (2001) also suggested a transition zone between the two realms from 28°N to 33°N latitude, based on floristic composition and the phylogeny and distribution of mammal species. The parapatry between *Hyla* clades even occurs in the Japanese islands, with *H. japonica* on the northern islands (Yakushima and northwards), and *H. hallowelli* (*arborea* group) on the southern islands (the Ryukus; IUCN, 2009).

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