

ELUCIDATION OF CRYPTIC DIVERSITY IN A WIDESPREAD NEARCTIC TREEFROG REVEALS EPISODES OF MITOCHONDRIAL GENE CAPTURE AS FROGS DIVERSIFIED ACROSS A DYNAMIC LANDSCAPE

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We investigate the evolutionary history of the wide-ranging Nearctic treefrog *Hyla arenicolor* through the integration of extensive range-wide sampling, phylogenetic analyses of multilocus genetic data, and divergence dating. Previous phylogeographic studies of this frog documented a potential signature of introgressive hybridization from an ecologically and morphologically divergent sister species. Based on our Bayesian phylogenetic analyses of mitochondrial DNA, we inferred strong phylogeographic structure in *H. arenicolor* as indicated by seven well-supported clades, five of which correspond to well-defined biogeographic regions. Clades from the Balsas Basin and southwestern Central Mexican Plateau in Mexico, and the Grand Canyon of Arizona, group with the morphologically, behaviorally, and ecologically divergent mountain treefrogs in the *H. eximia* group, rendering *H. arenicolor* as paraphyletic. The phylogenetic position of at least two of these three *H. arenicolor* clades within the *H. eximia* group, however, is most likely the result of several episodes of introgressive hybridization and subsequent mitochondrial gene capture separated in time and space, as supported by evidence from the nuclear genes. *Hyla arenicolor* from the Balsas Basin appear to be deeply divergent from other *H. arenicolor* and represent a distinctly different species. Results suggest that introgressive hybridization events, both ancient and contemporary, coupled with late Neogene vicariance and Pleistocene climate-driven range shifts, have all played a role in the historical diversification of *H. arenicolor*.

KEY WORDS: Ancient hybridization, biogeography, *Hyla*, introgression, mitochondrial gene capture, paraphyly, phylogeography.

Recent studies (reviewed in Schwenk et al. 2008) have documented numerous examples of introgressive hybridization between distinct species despite long-standing predictions of its rarity (Dobzhansky 1951; Mayr 1963). Insights from these studies suggest introgression is an important yet potentially misunderstood phenomenon in evolutionary biology. The history of intro-

gressed elements reveals valid components of species history, and its signature can provide insights into evolutionary processes that are otherwise difficult to detect. Ancient mitochondrial introgression, for example, can provide the sole remaining signature of past hybridization (Weisrock et al. 2005; McGuire et al. 2007; Bossu and Near 2009).

Introgressive hybridization in anurans has been particularly well documented (e.g., Blair 1972; Gerhardt 1974; Duellman and Trueb 1986; Lamb and Avise 1986), with several instances of mitochondrial gene capture reported (Lamb and Avise 1986; Smith and Green 2004; Chen et al. 2009). Maternally inherited mitochondrial DNA (mtDNA) frequently introgresses more rapidly than biparentally or paternally inherited components of a genome (Ballard and Whitlock 2004; Chan and Levin 2005), and mitochondrial genotypes characteristic of one species can thus occur against a predominantly nuclear background of another species (mitochondrial capture; Avise 2004). Mitochondrial gene trees may become especially misleading in cases in which introgressed haplotype lineages become fixed, leaving no hint of their heterospecific origin (Funk and Omland 2003). The smaller effective population size of mtDNA compared with nuclear loci may facilitate this process, such that even low levels of introgression may be sufficient to establish a neutral mitochondrial genotype in a foreign population (Takahata and Slatkin 1984; Ballard and Whitlock 2004; Chan and Levin 2005). Mitochondrial gene capture becomes detectable only when using multiple markers, such as morphology and molecular data from the nuclear genome.

In this study, we use multilocus data to examine the evolutionary history of a wide-ranging Nearctic anuran. The canyon treefrog, *Hyla arenicolor*, occupies rocky canyons and streams from Utah and Colorado in the United States south throughout most of mainland Mexico to southernmost localities in Oaxaca (Fig. 1). Previous research suggested the presence of at least three geographically structured mitochondrial lineages of *H. arenicolor* in the United States (Barber 1999a, b), one of which grouped with its sister species, the mountain treefrog (*H. eximia*). Mountain treefrogs in the *H. eximia* group (as defined herein) represent a monophyletic clade of four morphologically and ecologically similar species (*H. eximia*, *H. wrightorum*, *H. plicata*, and *H. euphorbiacea*; Smith et al. 2007) that are well differentiated from *H. arenicolor* based on both morphology (see Fig. 1) and call structure (Duellman 1970, 2001). Two of these species (*H. eximia* and *H. wrightorum*) are codistributed with *H. arenicolor* in pine-oak forests throughout much of their ranges (Fig. 1). The presence of one geographically localized clade of *H. arenicolor* within the *H. eximia* group has been considered evidence of cryptic diversity and suggestive of imperfect taxonomy (Barber 1999a), but this pattern could alternatively represent a signature of introgressive hybridization between *H. arenicolor* and a codistributed *H. eximia* group species. To date, however, no hybrids of these treefrogs have been reported despite studies that have examined morphology (Pierce 1968; Duellman 1970) and calls (Blair 1960; Pierce 1968; Duellman 1970; Renaud 1977; Sullivan 1986; Gergus et al. 2004), suggesting that interspecific hybrids are rare or nonexistent.

The clearest signature of introgression is the sympatric sharing of geographically localized genotypes between divergent

(morphologically or otherwise) species (Hare and Avise 1998; Masta et al. 2002). In recent or potentially ongoing hybridization, weakly divergent or indistinguishable introgressed haplotypes from one species are nested with haplotypes of another species. Ancient introgression is more difficult to detect, however, especially if haplotypes have sorted over time following introgression. As the time since last gene flow increases, introgressed lineages are more likely to be phylogenetically basal and less likely to show geographic associations with the donor species (Funk and Omland 2003). Although the phylogenetic association between otherwise divergent treefrogs inferred previously (Barber 1999a) provides a signature of introgressive hybridization, sampling limitations in that study preclude differentiation between introgression and imperfect taxonomy. Herein, we integrate extensive range-wide sampling of *H. arenicolor* with phylogenetic analyses of multilocus genetic data and divergence dating to confirm and explore the evolutionary history of introgression between *H. arenicolor* and the *H. eximia* group. If mitochondrial introgression has occurred, then nuclear data should contain a conflicting phylogenetic signal and instead reflect monophyly of the morphologically, behaviorally, and ecologically divergent *H. arenicolor*. Monophyletic cohesion of *H. arenicolor* based on nuclear data would then allow us to reject inferences of imperfect taxonomy. We seek to test this hypothesis with data from two mitochondrial genes and two nuclear loci, and further assess the informativeness of geographic patterns of genetic diversity despite a potential history of introgression.

Materials and Methods

TAXONOMIC SAMPLING AND DNA SEQUENCE DATA

We obtained tissues from 61 *H. arenicolor* from 56 localities representing the entire distribution (Fig. 1; Appendix 1). After preliminary analyses of mtDNA data indicated several lineages of *H. arenicolor* fell within the *H. eximia* group, we included six samples of the codistributed species *H. wrightorum*, five samples of codistributed *H. eximia*, and single representative samples of the allopatric species *H. plicata* and *H. euphorbiacea* in nuclear analyses to further assess potential introgression. Based on recent phylogenetic analyses (Faivovich et al. 2005; Wiens et al. 2005; Hua et al. 2009), we used *H. cinerea* and *H. versicolor* as outgroups.

We sequenced two mtDNA and two nuclear gene regions, including the mitochondrial genes NADH dehydrogenase subunit 4 and flanking tRNAs (ND4) and ATPase subunits 8 and 6 (ATPase), and nuclear loci proopiomelanocortin A (POMC) and β -crystallin (cryB). These mtDNA and nuclear gene regions have been previously shown to be informative at different levels of divergence within hylid frogs (ND4: Burns and Crayn 2006; POMC,

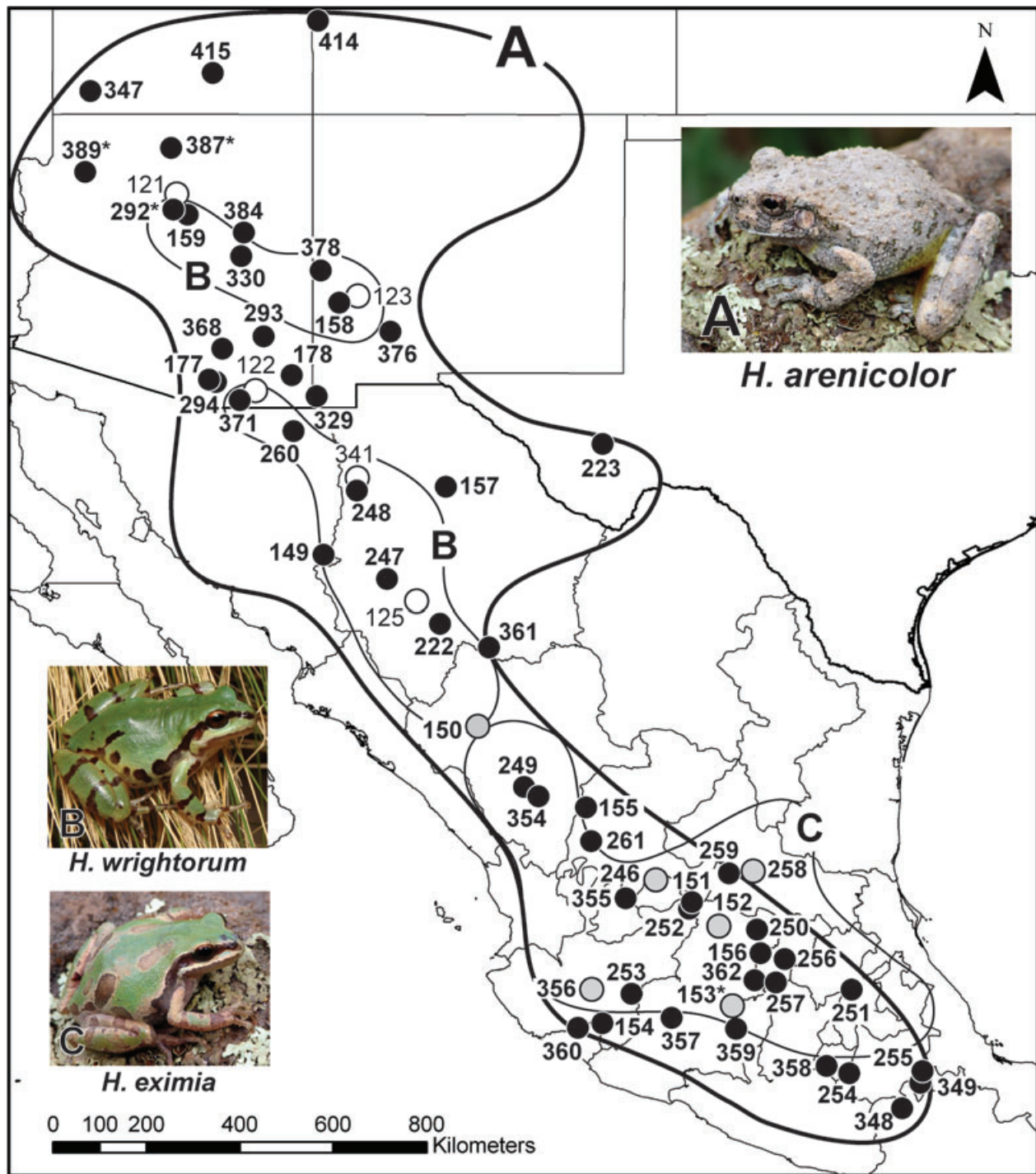


Figure 1. Generalized distribution map for (A) *Hyla arenicolor*, and the codistributed *H. eximia* group frogs (B) *H. wrightorum* and (C) *H. eximia*. Black dots indicate sampling localities for *H. arenicolor*, white dots indicate sampling localities for *H. eximia* group frogs, and gray dots indicate sampling localities in which both taxa were obtained. Numbers at dots refer to specific sample numbers of *H. arenicolor* (bold) and *H. eximia* group frogs (plain text) used in this study. Asterisks following some sample numbers denote multiple samples obtained from the same locality. The prefix "MX" was omitted from sample numbers for clarity.

cryB: Gamble et al. 2008; POMC: Wiens et al. 2005) or other amphibians (ATPase: Zajc and Arntzen 1999; Mahoney 2004). We extracted total genomic DNA from liver, toe clips, or tail clips (in the case of larvae) using the QIAGEN DNeasy Blood and Tissue

Kit (Qiagen, Valencia, CA), and amplified all gene regions in a mix containing 1.0 μ L of DNA, 6.25 μ L Takara ExTaq Polymerase Premix (Takara Mirus Bio Inc., Madison, WI), 4.25 μ L double-distilled water, and 0.5 μ L each primer (10 μ L). Primer

sequences were from Schauble et al. (2000; ND4), Wiens et al. (2005; POMC), and Dolman and Philips (2004; cryB), and for ATPase we used LysAF (5'-CAACCACCCTTGATGAATGCC-3') and C3FR (5'-GGGCTGGGGTTKACTATGTG-3'). We initiated PCR reactions by denaturing the DNA at 95°C for 2.5 min, with amplifications conducted in 35–40 cycles under the following conditions: denaturation at 95°C for 1 min, annealing at 50°C (ND4), 56°C (ATPase, cryB), or 58°C (POMC) for 1 min, and extension at 72°C for 1 min, followed by a final 10 min elongation at 72°C. Double-stranded PCR products were then checked by electrophoresis on a 1% agarose gel, purified using exonuclease and shrimp phosphatase, and sequenced in both directions with the amplification primers using a Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). For PCR products that yielded more than one band, as was the case for *H. wrightorum* and *H. eximia* amplified using cryB primers, we gel excised the appropriate sized band and purified the product using a QIAGEN QIAquick Gel Extraction Kit (Qiagen). We visualized sequences on an ABI Prism 3130 capillary autosequencer following removal of excess dyes from sequencing reactions using CentriSep spin columns (Princeton Separations, Inc., Adelphia, NJ).

For each sample, we edited and manually aligned sequences using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). Heterozygote sites in nuclear segments were identified when two different nucleotides were present at the same position in electropherograms of both strands, with the weakest peak reaching at least 50% of the strongest signal. When two or more heterozygote sites were identified in the same segment, we computationally determined the gametic phase of the variants using PHASE 2.1.1 (Stephens et al. 2001; Stephens and Donnelly 2003). PHASE uses Bayesian inference to reconstruct haplotypes and provides estimates of uncertainty associated with each prediction (Stephens and Donnelly 2003). For each nuclear dataset, five separate runs of 400 iterations each were carried out to ensure accurate haplotype estimation, with haplotype frequency estimates and goodness-of-fit values compared between runs. We excluded from phylogeographic analyses those haplotypes of *H. arenicolor* for which phase could not be determined with $\geq 90\%$ confidence. Initial analyses indicated that heterozygote positions unique to the comparatively few *H. eximia* group haplotypes were difficult to phase, so we set a confidence limit of $\geq 60\%$ for acceptance for these samples.

To detect intragene recombination within the two nuclear genes POMC and cryB, we used seven methods. Six of these methods were implemented using the program RDP2 (Recombination Detection Program) under default parameters (Martin et al. 2005). Two of the methods (RDP and Bootscan) are phylogenetic approaches that infer recombination when different parts of the genome result in discordant topologies, and four methods

(GENECONV, Chimaera, MaxChi, and SiScan) are nucleotide substitution approaches that examine the sequences either for a significant clustering of substitutions or for a fit to an expected statistical distribution. The seventh method was a four-gamete test (Hudson and Kaplan 1985) implemented in the program DnaSP version 5 (Librado and Rozas 2009) which scans the data for evidence of all combinations of bases between pairs of segregating sites. We considered recombination events that were detected by more than two of the methods as evidence for recombination.

PHYLOGENETIC ANALYSES

We analyzed mtDNA genes (which are inherited together as a single linkage unit) and nuclear genes separately, to avoid combining DNA sequences that may evolve at different rates (Bull et al. 1993) or that may have intrinsically conflicting phylogenetic histories (Edwards et al. 2007). We performed Bayesian phylogenetic analyses on the combined mtDNA dataset using MrBayes 3.1 (Ronquist and Huelsenbeck 2003). We conducted mixed-model analyses of the mtDNA data implementing separate models for each gene and the tRNA region. MrModeltest 2.1 (Nylander 2004) was used to select a best-fit model of evolution, based on Akaike information criteria (AIC), for each partition. Prior to final analyses, we explored the effects of branch length exponential priors (following McGuire et al. 2007) and temperature regimes for the Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses on convergence and mixing. A mean branch length prior of 100 (vs. 10 and 50) and temperature of 0.03 (vs. the default setting of 0.2) resulted in higher harmonic mean log likelihoods and better convergence and mixing.

Bayesian settings included random starting trees and default priors except the rate prior, which we set to “variable.” Analyses consisted of six runs (nrns = 6) each conducted with three heated and one cold Markov chain sampling every 100 generations for 5 million generations. Output parameters were visualized using the program TRACER version 1.4 (Rambaut and Drummond 2007) to ascertain stationarity and convergence of likelihood scores among runs. We further assessed convergence by evaluating posterior probability clade-support values postburnin using the online application Are We There Yet (AWTY, Wilgenbusch et al. 2004). Convergence occurred during the first 500,000 generations of each run; subsequently we conservatively discarded all samples obtained during the first one million (25%) generations as burnin. A 50% majority-rule consensus phylogram with nodal posterior probability support was estimated from the six runs postburnin, and we considered those nodes with $\geq 95\%$ posterior probability as strongly supported (Felsenstein 2004).

To evaluate relationships among alleles of the POMC and cryB markers, we created separate 95% statistical parsimony networks using the program TCS 1.21 (Clement et al. 2000). Network analyses of nuclear data can reveal multiple connections

between haplotypes and incorporate the possibility for the persistence of many ancestral haplotypes (Hare 2001; Posada and Crandall 2001). Networks can further help identify hybrid individuals (Joly and Bruneau 2006). We also created a concatenated dataset of the two nuclear genes to reconstruct phylogeny under Bayesian inference with MrBayes 3.1 as above. A mean branch length prior of 100 and a MCMC temperature prior of 0.03 resulted in good convergence and mixing. We treated multistate data as polymorphisms, and gaps as missing data. Convergence occurred during the first 500,000 generations of each run, and all samples obtained during the first one million (25%) generations were subsequently discarded as burnin.

DIVERGENCE DATING

We estimated divergence dates for *H. arenicolor* phylogroups from mtDNA data using a Bayesian relaxed molecular clock implemented in the program BEAST version 1.4.8 (Drummond and Rambaut 2007). The dataset was partitioned by gene, and best-fit models of evolution reestimated from the new dataset (see below) using MrModeltest. The partitioned BEAST .xml input file (available on request from the corresponding author) was created with BEAUti version 1.4.8 (Drummond and Rambaut 2007) and edited manually to allow parameters to be estimated independently among data partitions. We used an uncorrelated lognormal tree prior and node constraints obtained from the fossil record to estimate divergence dates throughout the tree. The final analyses consisted of two independent runs each of 50 million generations, with samples retained every 1000 generations, and with a Yule tree prior. TRACER was used to confirm acceptable mixing and likelihood stationarity of the MCMC chain, appropriate burnin, and adequate effective sample sizes (>200 for each estimated parameter) for each run and the combination of both runs. After discarding the first 5 million generations (10%) as burnin, we combined the trees and parameter estimates from the two runs using LogCombiner version 1.4.8 (Drummond and Rambaut 2007). We summarized the samples from the posterior on the maximum clade credibility tree using TreeAnnotator version 1.4.8 (Drummond and Rambaut 2007), with the posterior probability limit set to 0.5 and mean node heights summarized.

We implemented lognormal prior age distributions on calibration nodes. This allows for the placement of a “hard” minimum and “soft” maximum bound rather than two hard cutoff bounds (as with uniform distributions). Soft bounds can be structured to better account for uncertainty both in the taxonomic placement of fossils and in the dates assigned to fossils or geological events (Yang and Rannala 2006; Sanders and Lee 2007), giving low but nonzero probabilities to times outside the most likely age boundaries. A distribution with a long tail represents the diminishing probability that the divergence time may actually be

much older than the fossil with greater accuracy than an arbitrary oldest cutoff age. Allowing the analysis to sample states with lower probabilities will also result in larger confidence intervals, more accurately reflecting the error associated with divergence time estimates. To place calibration references on the tree, we sequenced several additional outgroups including the Eurasian *Hyla* species *H. annectans*, *H. chinensis*, *H. meridionalis*, *H. arborea*, *H. savignyi*, and *H. japonica*, and North American *Hyla* species *H. cinerea*, *H. squirella*, *H. andersonii*, *H. femoralis*, *H. gratiosa*, *H. avivoca*, *H. chrysocelis*, and *H. versicolor*. *Smilisca baudinii* and *Plectrohyla arborescendens* were used as outgroups to all *Hyla*. Because of potential problems associated with model parameter variance across heterogeneous datasets (Guiher and Burbrink 2008), we inferred divergence estimates for a reduced dataset, which included two individuals from each phylogenetic lineage of *H. arenicolor*, single individuals of *H. eximia*, *H. wrightorum*, *H. plicata*, and *H. euphorbiacea*, and the outgroup taxa. Before assigning fossil calibrations to nodes, we generated a Bayesian phylogram (not shown) using MrBayes to assess congruence between our topology and previously published hylid frog topologies based on multilocus datasets (Faivovich et al. 2005; Hua et al. 2009). The placement of two key taxa important for divergence dating (*H. squirella* and *H. gratiosa*) differed, and subsequently we chose to use two dating schemes in BEAST, as outlined below.

Sanchiz (1998) reported fossil *Hyla* closely related to extant *H. arborea* and *H. meridionalis* from the Lower Miocene of Austria about 17 million years ago (Mya). Following Smith et al. (2007), we placed this fossil at the most recent common ancestor (MRCA) of the European–Asian *Hyla* clade, with a zero offset (hard lower bound) of 17 Mya, a median of 20 Mya, and a lognormal standard deviation of 0.42, producing a 95% prior credible interval spanning from 18.5 Mya to the beginning of the Miocene 23 Mya. *Hyla miocenica*, an extinct frog from the early Barstovian North American Land Mammal Age (NALMA) of the Miocene (14–16 Mya; Holman 2003), is similar to *H. chrysocelis* and *H. versicolor*. In our Bayesian analysis, *H. avivoca*, *H. chrysocelis*, and *H. versicolor* form a shallow clade of uncertain relationships; thus, we placed the fossil calibration at the MRCA of this clade, with a zero offset of 14 Mya, a median of 15 Mya, and a lognormal standard deviation of 0.42, producing a 95% prior credible interval spanning from 14.5 to 16 Mya. In addition to *H. miocenica*, two other North American pre-Pleistocene fossil *Hyla* have been reported: *H. goini*, from the Miocene Hemingfordian NALMA (15–19 Mya; Holman 1967, 2003), and *H. miofloridana*, also from the Miocene Hemingfordian NALMA (Holman 2003). *Hyla goini* is closely related to extant *H. squirella*, and *H. miofloridana* is similar to *H. gratiosa* (Holman 2003). In the preliminary Bayesian analysis, we found that *H. gratiosa* and *H. cinerea* formed a well-supported clade

sister to all other North American *Hyla* except *H. squirella*, which was well supported as the sister to the *H. gratioiosa*-*H. cinerea*/other North American *Hyla* clade. Previous studies based on multiple loci, however, strongly inferred a *H. squirella*-*H. cinerea*-*H. gratioiosa* clade (Faivovich et al. 2005; Hua et al. 2009). We therefore constrained *H. squirella*, *H. cinerea*, and *H. gratioiosa* to form a monophyletic clade in our BEAST analyses, and placed the fossil calibration at the MRCA of this clade, with a zero offset of 15 Mya, a median of 17 Mya, and a lognormal standard deviation of 0.42, producing a 95% prior credible interval extending from 16 Mya to the beginning of the Hemingfordian NALMA 19 Mya. To test the effect of this constraint on our estimates of divergence dates, we also ran analyses without this calibration.

Results

DNA SEQUENCE DATA

Alignment for both mitochondrial gene regions was straightforward, and no indels were present. The ND4 (759 base pairs, bp, total: 688 bp fragment of ND4, 71 bp of the flanking tRNA genes) and ATPase (839 bp total: 165 bp ATPase 8, 684 bp ATPase 6, with an overlap of 10 bp between the two genes) genes were sequenced for all individuals. Models of sequence evolution selected for the mtDNA partitions were GTR + I + G (ND4, ATPase 6) and HKY + G (tRNAs and ATPase 8). The models selected for the concatenated nuclear dataset were GTR + I + G (POMC) and GTR + I (cryB). All sequences were deposited in GenBank (accession numbers HM152030–HM152474), and our data matrices were made available on the TreeBase website.

Alignment for POMC (520 bp) was straightforward, and no indels were present. We were unable to obtain sequence data for four individuals (MX251, MX259, MX292, and MX330). Haplotype frequency estimates and goodness-of-fit values across all runs in PHASE were similar. A large number of individuals sequenced (44 out of 68) contained heterozygous positions, and the gametic phase for many of these variants was difficult to infer with $\geq 90\%$ (*H. arenicolor*) or $\geq 60\%$ (*H. eximia* group) confidence. As a result, we excluded sequences for 13 *H. arenicolor*, two *H. wrightorum*, and two *H. eximia* from network analyses. The final dataset for these analyses contained 29 heterozygous individuals, represented by two phased alleles, and 22 homozygous individuals represented by single alleles.

Amplification of cryB produced products of two different sizes (167 or 168) in *H. arenicolor* caused by a 1 bp indel. *Hyla eximia* sequences contained a 12 bp indel. We were unable to obtain sequence data for three individuals (MX259, MX292, and MX330). Haplotype frequency estimates and goodness-of-fit values across all runs in PHASE were similar, and 24 of the 69 individuals were heterozygous. The gametic phase of one *H. arenicolor*

haplotype could not be confidently phased, so we discarded it. The final dataset for network analyses contained 23 heterozygous individuals represented by two phased alleles, and 45 homozygous individuals represented by single alleles.

None of the six methods used in RDP2 detected recombination in the POMC or cryB datasets. The four-gamete test, however, detected a minimum of eight recombination events in the POMC dataset and five recombination events in the cryB dataset. This test relies on the infinite-sites model (i.e., only one mutation per nucleotide site), and recurrent mutations at the same nucleotide site during the genealogical history of a sample may be driving the detection of inferred recombination events.

PHYLOGENETIC ANALYSES

We inferred strong phylogeographic structure in *H. arenicolor* from our Bayesian analyses of mtDNA as indicated by seven well-supported clades (Fig. 2). Five of these clades largely correspond to well-defined biogeographic regions characterized by prominent phylogeographical breaks (e.g., Jaeger et al. 2005; Riddle and Hafner 2006), and include the Sonoran Desert (SD) and associated highlands, Chihuahuan Desert (CD) and associated highlands, Colorado Plateau (CP), Central Mexican Plateau (CMP), and Balsas Basin (BB). The two other clades represent *H. arenicolor* from the southwestern region of the Central Mexican Plateau (CMP-SW), and *H. arenicolor* from the Grand Canyon of Arizona (GC). These latter two clades are nested within the *H. eximia* group, rendering *H. arenicolor* as nonmonophyletic, with *H. arenicolor* from the BB forming the basal clade (or sister clade, see Discussion) to the entire *H. eximia* group.

Within the “core” *H. arenicolor* clade (those not associated with *H. eximia* group), there is strong support for a deep split between the SD clade and the combined CP, CD, and CMP clades. Average uncorrected pairwise divergences between these groups (SD/CP + CD + CMP), as determined using the program MEGA 4 (Kumar et al. 2008), is 7.4%. A subsequent split separates the CP clade from the CD and CMP clades. The average uncorrected pairwise divergence between these groups (CP/CD + CMP) is 3.0%. The CD and CMP clades diverge at a more shallow level, with an average uncorrected pairwise divergence between these clades of 1.4%.

The three clades of *H. arenicolor* associated with the *H. eximia* group occupy different positions in the phylogenetic tree. Their deep sequence divergences from core *H. arenicolor* (BB, 13.8%; CMP-SW, 13.3%; GC, 13.4%) combined with strongly supported monophyly and structure within the *H. eximia* group suggest that this pattern is not a result of recent incomplete lineage sorting (Avice and Ball 1990). A basal split separates the BB clade from all *H. eximia* group frogs. *Hyla arenicolor* from the CMP-SW are strongly supported as the sister clade to several *H. eximia* group species, including

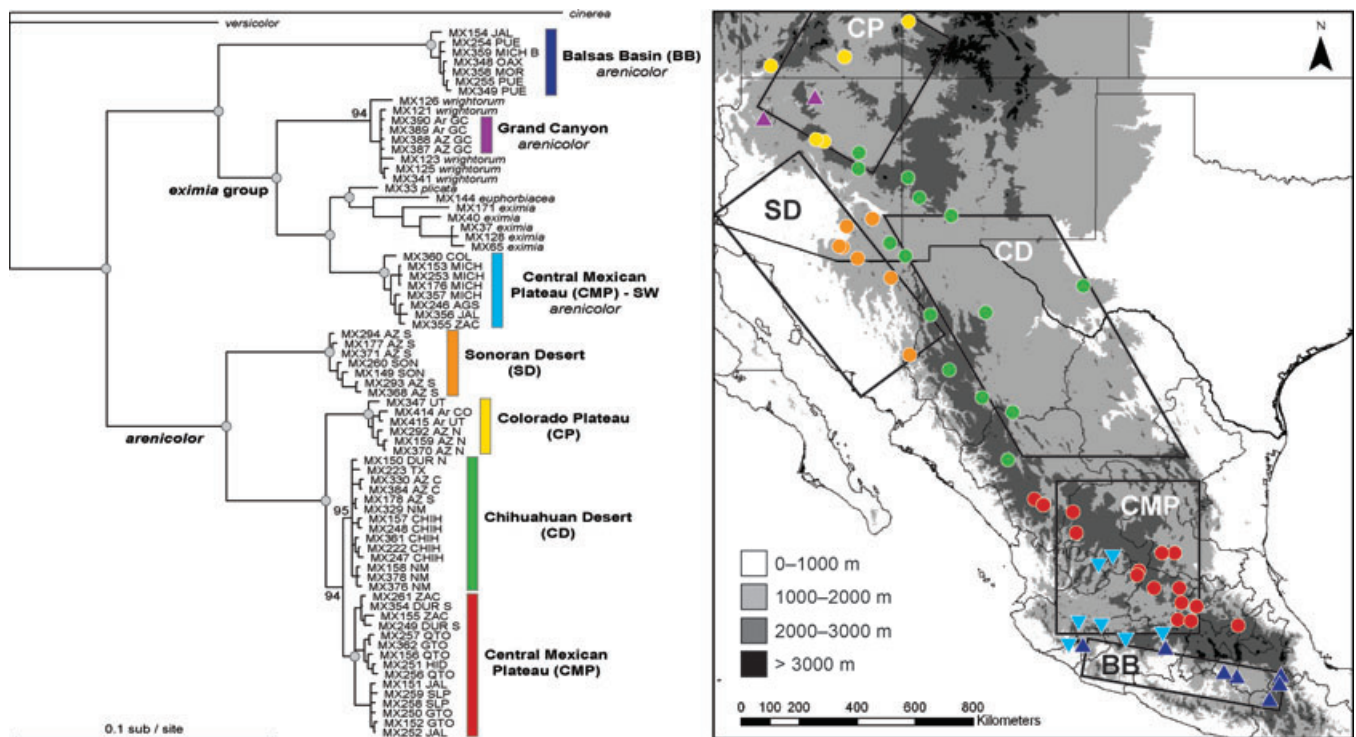


Figure 2. Phylogenetic estimate for *Hyla arenicolor* based on mixed-model Bayesian analyses of mitochondrial DNA sequence data. Major clades are individually color coded. Numbers at nodes indicate % posterior probability values; gray-filled circles represent nodes that received 100% posterior probability support. Map depicts the distribution of color-coded haplotypes, with generalized areas of major biogeographic regions delineated by parallelograms. Triangles represent haplotypes from clades found to be associated with the *H. eximia* group.

H. plicata, *H. eximia*, and *H. euphorbiacea*. The *H. arenicolor* from the GC are nested within *H. wrightorum*.

Statistical parsimony network analyses for POMC and *cryB* (Fig. 3) displayed similar patterns. For visual clarity, we set haplotype connections in the POMC network analyses to four mutational steps within the 95% connection limit. Within this framework, *H. arenicolor* POMC haplotypes formed four networks. Contained within the individual networks are: *H. arenicolor* haplotypes from the BB (Network 1); all *H. arenicolor* haplotypes from the SD and some *H. arenicolor* haplotypes from the CD (Network 2); most *H. arenicolor* haplotypes from the CP and all those from the GC (Network 3); and a large network of *H. arenicolor* haplotypes primarily from the CD, CMP, and CMP-SW (Network 4). Within Network 4, most *H. arenicolor* haplotypes from the CMP-SW were clustered together. One allele from a heterozygous *H. wrightorum* was also contained within Network 4; all other *H. eximia* group haplotypes were contained in separate networks. Details on individual haplotypes found within each POMC network can be found in the Table S1.

Statistical parsimony analyses of the *cryB* gene grouped most *H. arenicolor* into two networks. All *H. arenicolor* from the BB

were contained within one network (Network 1), whereas all other *H. arenicolor* (representing samples from all other major mtDNA clades) formed the second (Network 2). Within this network, most *H. arenicolor* haplotypes from the SD and CMP-SW formed independent clusters. Homozygous haplotypes from two *H. arenicolor*, one from the CP (Utah) and one from the SD (Santa Rita Mountains, AZ), were identical to several *H. wrightorum* and clustered inside a larger network of *H. wrightorum*. Interestingly, these two *H. arenicolor* are from localities outside of the known range of *H. wrightorum*. Details on individual haplotypes found within each *cryB* network can be found in the Table S2.

Bayesian analyses of the combined nuclear genes (Fig. 4) inferred several well-supported monophyletic lineages largely congruent with mtDNA analyses (Fig. 5), but otherwise recovered little supported structure. Instances of discordance were confined to the phylogenetic placement of three haplotypes near the edge of mtDNA clade boundaries (Fig. 4). The three individuals with heterospecific haplotypes identified in network analyses (*H. arenicolor* with *H. wrightorum* alleles, MX294 and MX347; *H. wrightorum* with *H. arenicolor* alleles, MX122) were excluded from analyses. The topology was characterized by

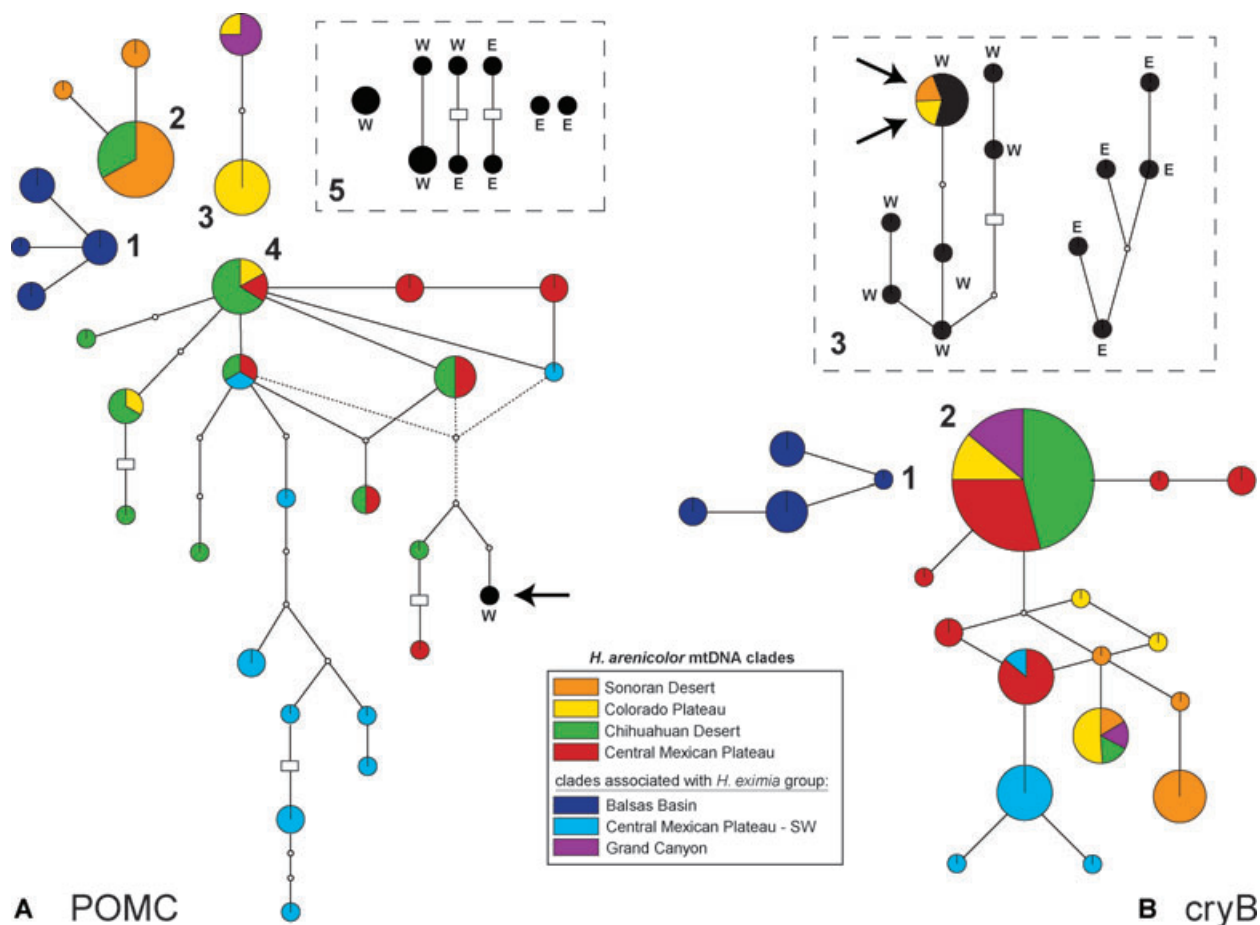


Figure 3. Maximum parsimony network analyses for the two nuclear genes (A) POMC and (B) cryB. For visual clarity, haplotype connections in the POMC network analyses were set to four mutational steps within the 95% connection limit. A circle represents each haplotype and the size of each circle is proportional to the frequency of the haplotypes in the dataset. Network ambiguities are designated by dashed lines and unsampled alleles are represented by white dots. Each branch represents one mutational step between haplotypes; white blocks indicate four mutational steps. Networks and haplotypes within dashed-line boxes belong to codistributed frogs in the *Hyla eximia* group (*w*, *H. wrightorum*; *e*, *–*). Haplotypes are color coded to correspond to the major mitochondrial clades listed in inset. Note the inclusion of *H. arenicolor* from the southwestern Central Mexican Plateau and Grand Canyon in networks with other *H. arenicolor*, and not with haplotypes of the codistributed *H. eximia* group. Instances of nuclear introgression between *H. arenicolor* and *H. wrightorum* indicated by arrows. Haplotypes found in each numbered network or box can be found in the tables in Supporting information.

well-supported, monophyletic clades of *H. arenicolor* from the CP and the CMP-SW that, together with a weakly-supported, monophyletic clade from the SD and *H. arenicolor* from the CD and CMP, formed a well-supported monophyletic group. All *H. arenicolor* from the GC, indistinguishable from *H. wrightorum* based on mtDNA analyses, grouped with other *H. arenicolor* from the CP. Weakly supported as sister to this group is a clade of *H. wrightorum* and *H. eximia*. *Hyla arenicolor* from the BB formed a strongly supported monophyletic group, but interestingly appear as sister to the *H. wrightorum*-*eximia* + *H. arenicolor* clade. A weakly supported clade of *H. plicata* and *H. euphorbiacea* form the sister clade to the BB + (*H. wrightorum*-*eximia* + *H. arenicolor*) clade.

DIVERGENCE DATING

Selected models of sequence evolution for each partition in the BEAST analyses were GTR + I + G (ND4, ATPase 6), SYM + G (approximated by the GTR + G model; tRNAs), and HKY + I + G (ATPase 8). Posterior estimates of divergence times with the calibrated and constrained *H. sierrae-cinerea-gratiola* clade (Fig. 6) resulted in posterior mean ages and 95% highest posterior density intervals (HPD) similar to estimated dates of about 30–40 Mya for the origin of North American *Hyla* based on divergence dating of multilocus sequence data for all hylid frogs (Wiens et al. 2006) and the fossil record (Holman 2003; but see Sanchiz 1998). Estimates without this calibration resulted in considerably older posterior mean ages

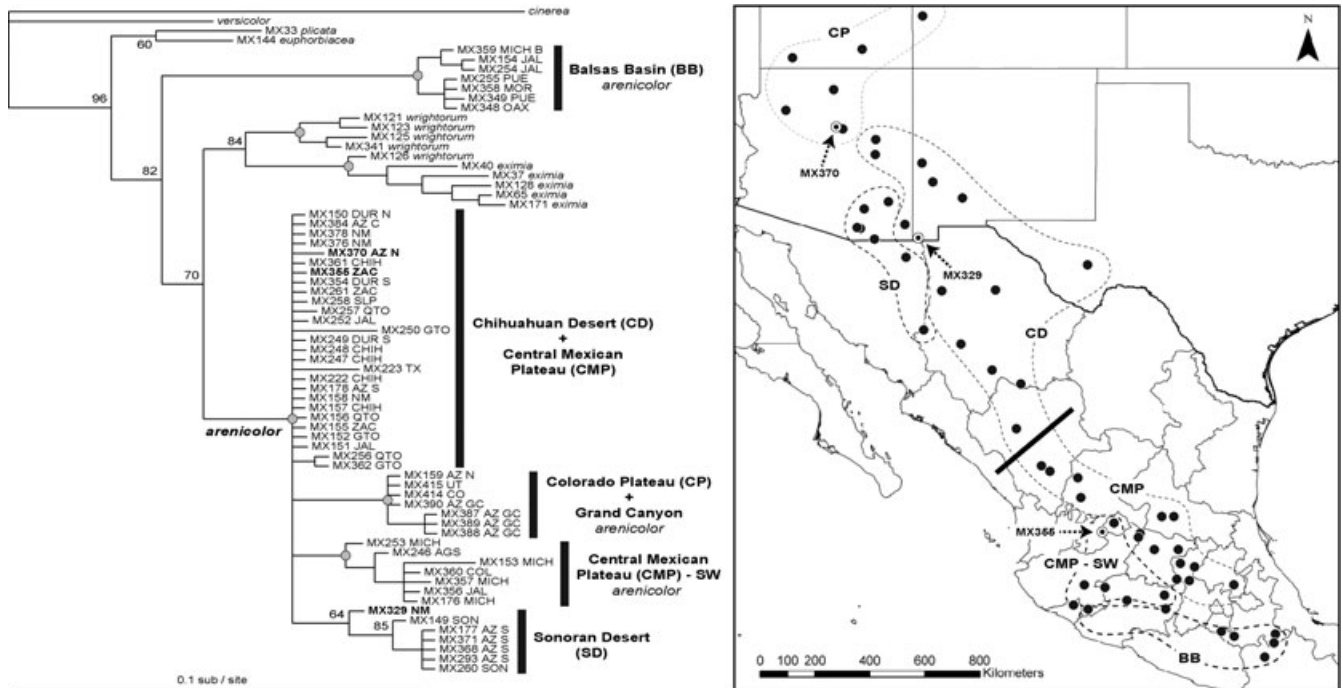


Figure 4. Phylogenetic relationships among major lineages of *Hyla arenicolor* based on Bayesian analyses of concatenated nuclear sequences. Numbers at nodes indicate % posterior probability values; gray-filled circles represent nodes that received 100% posterior probability support. Instances of discordance between the nuclear and mitochondrial DNA data are identified by arrows on map, and phylogenetic position of discordant haplotypes in nuclear tree typed in bold font. Approximate distribution of inferred clade boundaries based on both datasets delineated with dashed lines on map. Thick dark line near center of map denotes the inferred recent divergence of the Chihuahuan Desert and Central Mexican Plateau clades based on mtDNA.

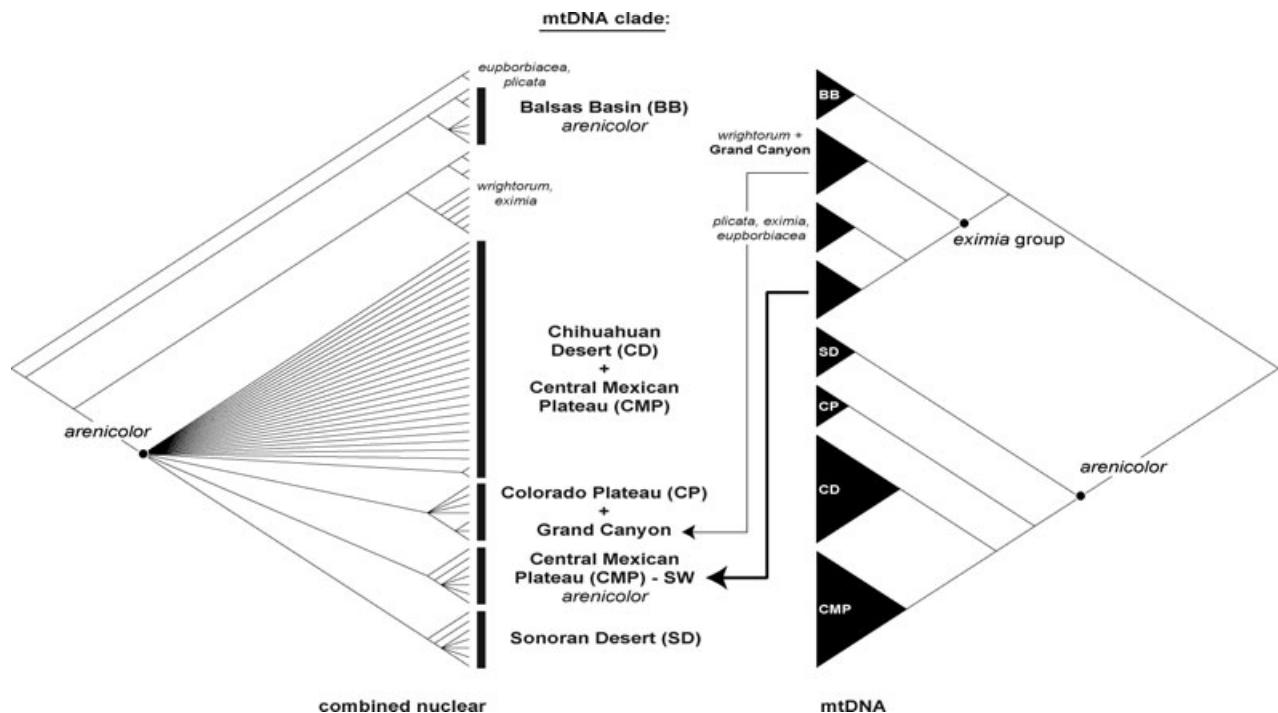


Figure 5. Comparison of simplified phylogenetic trees based on Bayesian analyses of concatenated nuclear (right) and mitochondrial DNA sequence data. Black dots represent relevant nodes that received 100% posterior probability support. Note the alternative placement of *H. arenicolor* from the southwestern Central Mexican Plateau and Grand Canyon in the mtDNA tree in a monophyletic group of *H. arenicolor* in the nuclear tree.

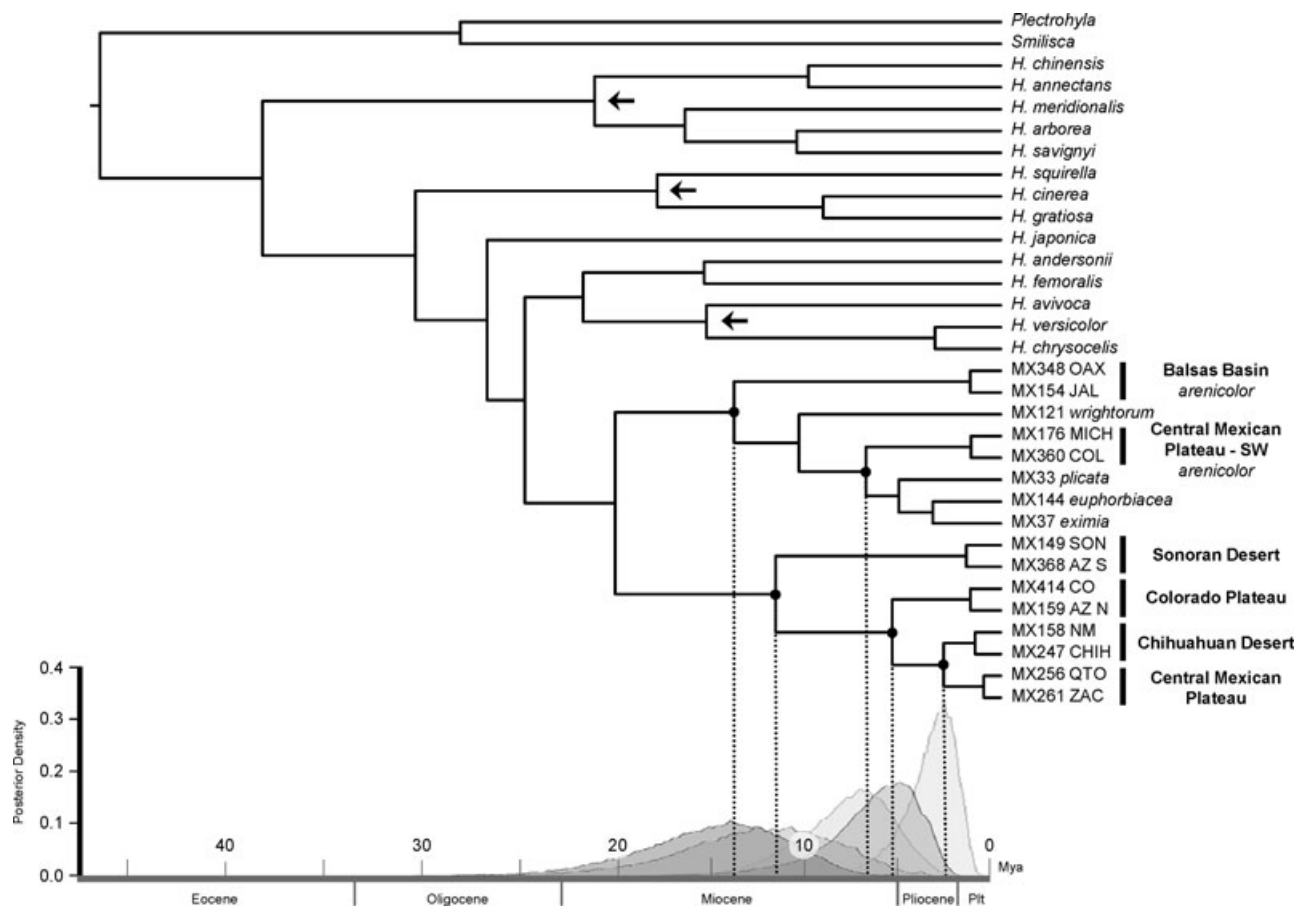


Figure 6. Chronogram with estimated divergence times for major clades of *Hyla arenicolor* inferred using a Bayesian relaxed clock model. Bayesian posterior density plots of estimated time to most recent common ancestor for major clades are depicted below tree. Arrows denote placement of fossil calibrations detailed in text, and “Plt” refers to Pleistocene.

and 95% HPD. We therefore consider our estimates with a constrained *H. squirella-cinerea-gratiosa* clade calibration to be appropriate, but note the caveats associated with the uncertainties of molecular dating (e.g., Pulquério and Nichols 2007; Lee et al. 2009).

Divergence dating estimates based on mtDNA data suggest relatively ancient divergences (Fig. 6; Table 1). The posterior mean estimate for the initial divergence between the *H. eximia* group (and associated *H. arenicolor*) and core *H. arenicolor* is about 22 Mya with a broad 95% HPD (Table 1). Within the core *H. arenicolor* clade, diversification may have begun as early as the Middle Miocene and terminated in the Pliocene. The two monophyletic *H. arenicolor* clades within the *H. eximia* group appear to have diverged from other *H. eximia* at two different times during the Miocene. As noted previously, *H. arenicolor* from the GC are indistinguishable from *H. wrightorum* based on our mtDNA sequence data, suggesting a very recent or ongoing divergence.

Table 1. Estimated divergence dates among groups of *Hyla arenicolor* based on relaxed phylogenetic analyses of mitochondrial DNA using lognormal prior distributions on fossil calibrations. Posterior mean ages and 95% highest posterior density intervals (HPD) provided in millions of years ago (Mya). SD, Sonoran Desert; CD, Chihuahuan Desert; CP, Colorado Plateau; CMP, Central Mexican Plateau; BB, Balsas Basin; CMP-SW, southwestern Central Mexican Plateau.

Node	Posterior mean age (95% HPD)
SD/CP+CD+CMP	11.4 (4.4, 19.3)
CP/CD+CMP	5.5 (1.6, 10.7)
CD/CMP	2.9 (0.5, 6.2)
core <i>H. arenicolor</i> / <i>H. eximia</i> group (plus associated <i>H. arenicolor</i>)	19.6 (11.6, 29)
BB/ <i>H. eximia</i> group	13.5 (6.2, 21.7)
CMP-SW/ <i>H. eximia</i> group (minus <i>H. wrightorum</i>)	6.8 (2.4, 12.1)

Discussion

PARAPHYLY, INTROGRESSION, AND THE BALSAS BASIN LINEAGE

In agreement with a previous study (Barber 1999a), we inferred a paraphyletic *H. arenicolor* based on our phylogenetic analyses of mtDNA, with three geographically disparate clades falling outside of a main core group of *H. arenicolor*. Potential causes of mtDNA paraphyly are varied (Funk and Omland 2003), but include incomplete lineage sorting, introgression, and imperfect taxonomy. Although incomplete lineage sorting is typical of shallow species trees with closely related taxa recently diverged in time (Avice 2000), some combinations of branching patterns and branch lengths deep in time can cause lineages to sort in a way in the past that violates monophyly in the present (Degnan and Rosenberg 2009). This phenomenon, however, requires short branches deep in the tree, reflective of ancient rapid radiations (Edwards et al. 2005; Degnan and Rosenberg 2009). If the phylogenetic placement of the three clades of *H. arenicolor* associated with the *H. eximia* group were caused by a mis-sorting of ancestral polymorphisms deep in time, branches near the base of the *H. eximia* group tree and time between divergences should be short. This is not reflected by our topology (Fig. 2) or chronogram (Fig. 6). Several lines of evidence suggest the phylogenetic nesting of *H. arenicolor* from the CMP-SW and the GC in the *H. eximia* group is instead most likely a result of separate instances of mitochondrial introgression. *Hyla arenicolor* from the CMP north to the CP are morphologically indistinguishable with a similar call type (Pierce 1968; Duellman 1970, 2001), and com-

prise a well-supported monophyletic clade based on our nuclear data.

The currently identified *H. arenicolor* from the BB, however, appear to be deeply divergent from both other *H. arenicolor* and *H. eximia* group species. Although morphological similarities unite this lineage with other *H. arenicolor* (Pierce 1968; Duellman 1970, 2001), call and adult size differences indicate that the BB lineage may in fact represent a distinct species (Pierce 1968). Phylogenetic analyses based on our nuclear data do not group BB frogs with other *H. arenicolor*, supporting an inference that imperfect taxonomy may be the cause of the observed paraphyly in *H. arenicolor* with respect to the BB lineage. The BB lineage thus appears to be a new species that awaits formal description.

INTROGRESSION AND MITOCHONDRIAL CAPTURE

The phylogenetic position of the CMP-SW and GC clades of *H. arenicolor* within the *H. eximia* group based on mtDNA data is most likely the result of two distinct episodes of introgressive hybridization and subsequent mitochondrial gene capture separated in time and space (Fig. 7). In at least one and possibly both of these instances, these events were followed by reproductive isolation, resulting in divergent, monophyletic, and geographically structured mtDNA lineages. Divergence date estimates based on mtDNA sequence data provide insight into the timing of introgressive hybridization and mitochondrial gene capture, and suggest a relatively ancient first event that most likely occurred in the Late Miocene between *H. arenicolor* distributed across the southwestern region of the CMP and *H. eximia* group frogs. *Hyla arenicolor*

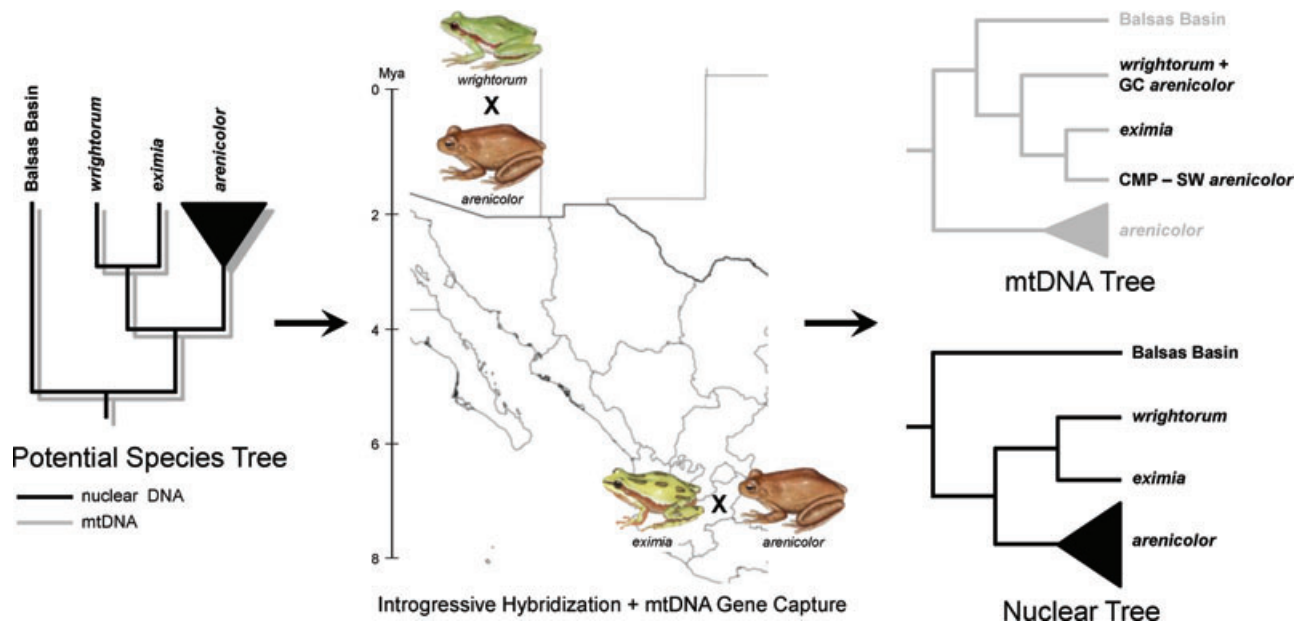


Figure 7. Simplified diagram of hypothesized evolutionary events leading to observed discordance between mitochondrial and nuclear gene trees. The phylogenetic position of the southwestern CMP (CMP-SW) and Grand Canyon (GC) clades of *H. arenicolor* within the *H. eximia* group (upper right) is most likely the result of two distinct episodes of introgressive hybridization and subsequent mitochondrial gene capture separated in time and space.

and *H. eximia* are codistributed throughout much of the CMP, including the southwestern portion, and several of our samples of both species were collected from the same locality (Fig. 1). As noted previously, no morphological hybrids are known, and in expanded sampling of *H. eximia* (data not shown), we found no evidence of recent hybridization as would be indicated by *H. arenicolor* haplotypes nested within *H. eximia* phylogroups. Ancient hybridization followed by reproductive isolation thus appears to be a strong explanation for this pattern. In classic stepwise fashion, following mitochondrial capture and subsequent reproductive isolation, introgressed *H. arenicolor* would develop mtDNA mutations independent of *H. eximia* group frogs through time, and eventually acquire a divergent and reciprocally monophyletic signature. Although our sampling across the entire CMP was geographically widespread (Fig. 2), it remains unclear if the distributions of the CMP-SW and main CMP clades are in fact separated, or if sequencing additional samples will reveal more introgressed *H. arenicolor* in the main CMP.

The recent mitochondrial introgression in the GC *H. arenicolor* clade is seemingly complex. Our sampling and previous studies (Barber 1999a, b) indicate mtDNA haplotypes of *H. arenicolor* from throughout the canyon are identical to *H. wrightorum*, suggesting very recent or ongoing hybridization and gene capture, and not a repeated pattern of ancient hybridization followed by isolation (as shown with the other introgressed clade of *H. arenicolor*). This suggests that either the GC was colonized very recently from an introgressed population of *H. arenicolor*, or *H. arenicolor* and *H. wrightorum* are exchanging genes across a hybrid zone in the GC. Surveys in the GC, however, have failed to document the occurrence of the highland-adapted *H. wrightorum* (Suttkus et al. 1976; Miller et al. 1982). A more plausible explanation is that introgressive hybridization occurred during the Pleistocene, possibly around the Mogollon Rim, following a range contraction of the CP clade of *H. arenicolor*, with subsequent post-Pleistocene colonization of the GC. This would explain the presence of *H. wrightorum* nuclear alleles in the southwestern Utah sample of *H. arenicolor*—a locality over 230 km away from the nearest *H. wrightorum* population and across the GC.

The observed patterns of asymmetrical mitochondrial introgression suggest that the *H. eximia* group mitochondrial genome was transferred into *H. arenicolor* on at least two occasions. Primary hybridizations most likely occurred between male *H. arenicolor* and *H. eximia* group females rather than the reciprocal. Mitochondrial introgression would then be mediated by hybrids that transmit their maternal *H. eximia* group mtDNA to the paternal *H. arenicolor* gene pool via backcrosses with *H. arenicolor* males. Provokingly, our data show some limited evidence for bidirectional nuclear introgression between *H. arenicolor* and a *H. eximia* group frog (*H. wrightorum*) as well, although this is perhaps less common. Of the three *H. wrightorum* included in

POMC network analyses, one allele from a heterozygous individual clustered with other *H. arenicolor* (Fig. 3A). In the cryB network analyses, two *H. arenicolor* haplotypes (MX294, MX347) from localities outside of the known range of *H. wrightorum* were identical to *H. wrightorum* (Fig. 3B). At least in these cases, the allopatric populations (see Fig. 1) suggest the retention of introgressed nuclear alleles and not first-generation hybridization events.

Hyla arenicolor is distributed across a broad latitudinal range with large elevational potentials within regions that have experienced great climatic shifts and oscillations through time. A plausible explanation for the observed patterns of introgression within *H. arenicolor* is that occasional hybridizations may have been followed by selective sweeps based on thermal adaptation of favorable mitochondria (Somero 2002; Ballard and Whitlock 2004). Other explanations for mitochondrial gene capture, such as neutral genetic drift following hybridization (Ballard 2000; Ballard and Whitlock 2004), cannot be ruled out, but seem less likely given the two temporally divergent events observed leading to monophyletic, geographically structured lineages across a rather large landscape.

EVOLUTIONARY HISTORY OF *H. ARENICOLOR*

The evolutionary history of *H. arenicolor* has been marked by temporally and spatially distinct episodes of introgressive hybridization and mitochondrial gene capture, yet geographic patterns of genetic diversity are still discernable. Basal divergences in the mtDNA tree and estimated divergence dates suggest the diversification of *H. arenicolor* began as early as the mid-Miocene. An inferred initial divergence between *H. arenicolor* east and west of the Sierra Madre Occidental suggests *H. arenicolor* was widespread throughout western North America by this time. This relatively ancient east–west split has been documented in a number of codistributed taxa (e.g., Orange et al. 1999; Jaeger et al. 2005). Following this initial divergence, two more splitting events are traceable. In the north, the CP clade diverged from *H. arenicolor* distributed throughout the CD and CMP, most likely in the Late Miocene. To the south, the CD and CMP clades appear to have diverged in the Pliocene, prior to the onset of more extreme glacial–interglacial cycles.

The impact of Pleistocene glaciations on the genetic diversity and distributions of species distributed across western North America is varied, but general trends of range expansions and contractions are evident (e.g., Jaeger et al. 2005; Douglas et al. 2006; Castoe et al. 2007). Previous research utilizing fine-scale genetic sampling (Barber 1999b) suggests that at least one clade of *H. arenicolor* (from the CD) has recently expanded. As a result, it appears that areas of contact and gene flow between otherwise evolutionarily divergent clades have developed. These observations provide a likely explanation for the sharing of mtDNA

clade-specific alleles in our network analyses of nuclear sequences, and the alternative placements of haplotypes in our two gene trees. For example, three *H. arenicolor* within our CD mtDNA clade collected along the western periphery of the CD have POMC alleles contained within the SD network (Table S1), and one is placed in the SD clade of our concatenated nuclear tree. One *H. arenicolor* within our CP mtDNA clade from the southern portion of the CP is placed with CD and CMP haplotypes (POMC network, concatenated nuclear tree). Gene flow may be a less likely explanation for the sharing of POMC alleles between the CD and CMP mtDNA clades, as individuals carrying identical POMC alleles are randomly distributed within the ranges of the mtDNA-defined clades rather than concentrated near geographic points of contact, a pattern predicted by incomplete lineage sorting (Hare and Avise 1998). A more definitive assessment of gene flow among these mtDNA lineages, however, requires the evaluation of additional multilocus genetic data and denser sampling at mtDNA lineage contact zones.

EVOLUTIONARY IMPLICATIONS

Results from our study are broadly significant. Here we have demonstrated a spatially and temporally dynamic pattern of unidirectional mitochondrial introgression and gene capture between two groups of frogs with a historical documentation of strong morphological and call-related divergences and an apparent lack of current hybrids. Introgressive hybridization events, both ancient and contemporary, have likely played a role in the diversification of *H. arenicolor*, yet have led to a potentially misleading mtDNA gene tree. Ancient hybridizations and mitochondrial gene capture, followed by reproductive isolation, may result in the establishment of a new evolutionary trajectory in the introgressed lineage over time and the development of new clade-specific mutations. This is perhaps an alternative explanation for “mtDNA fossils” (Bossu and Near 2009), which posits mitochondrial gene capture from an extinct ancestor as a means for explaining ancient introgressed lineages distantly related to extant lineages.

We uncovered evidence for both mitochondrial and nuclear introgression in our study which, in agreement with a growing body of literature, highlights the necessity of multilocus data when investigating the evolutionary history of closely related species or taxonomic groups such as anurans with a propensity for hybridization. A recent debate has arisen on the utility of mtDNA in avian phylogeographic studies (Barrowclough and Zink 2009; Edwards and Bensch 2009). Mitochondrial gene sequences are well known to have an appreciably higher probability of detecting geographical or taxonomic limits within recently evolved groups than DNA sequences from nuclear genes (Moore 1995; Hudson and Coyne 2002; Zink and Barrowclough 2008). Accordingly, as well argued by Barrowclough and Zink (2009), the utility of mtDNA as an evolutionary marker to infer recent geographical patterns is robust.

Nuclear markers may also be necessary, however, for more than coupling evolutionary processes with pattern. Although not avian in taxonomic focus, results from our study on a wide-ranging hylid frog suggest reliance on mtDNA would lead to erroneous assumptions about the underlying species tree. With the addition of relatively few nuclear markers, we obtained the necessary insight to infer the probable causes of the misleading mtDNA pattern. Although the use of only a small number of nuclear markers with potentially different evolutionary histories may result in less phylogenetic accuracy and confidence (Maddison and Knowles 2006; Edwards et al. 2007), in our study this approach resulted in a cost-effective cross-examination of a potentially misleading mtDNA gene tree.

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Appendix

Collection and voucher data for genetic samples. Sample identification is underlined and follows voucher information in parentheses. Museum codes follow Leviton et al. (1985). Nonstandard abbreviations are as follows: MZFC, Museo de Zoología ‘Alfonso L. Herrera’, Facultad de Ciencias, Universidad Nacional Autónoma de México; UANL, Universidad Autónoma de Nuevo León; ANMO, Adrián Nieto-Montes de Oca; EMO, Eric O’Neill; FRMP, Fernando Ricardo Mendoza-Paz; FMQ, Fernando Mendoza-Quijano; HL, Wen Hao Chou field series; JAC, Jonathan A. Campbell; JJ, Jeremy Jungles; KK, Katy Klymus; OFV, Oscar Flores-Villela; PB, Paul Barber; RWB, Robert W. Bryson, Jr.; SAS, Sarah A. Smith.

HYLA ARENICOLOR

MEXICO: *Aguascalientes*: W La Congoja, Sierra Fria (RWB 0699, MX246). *Chihuahua*: Rancho El Nido, Sierra del Nido (RWB 07276, MX157); N Creel (RWB 07293, MX247); E Mesa de Agostadero (RWB 07328, MX222); 1.3 mi E (by road) Chuhuichupa (MVZ 133015, MX248); approx. 23 km S Hidalgo de Parral on Hwy 45 (JAC 29275, MX361). *Colima*: Agua Fria, close to Minatitlan off Hwy 98 (JAC 28278, MX360). *Durango*: SE Mesa de las Navar (RWB 07322, MX150); Rancho Santa Bárbara (FRMP 171, MX249); off rd. from Cd. Durango south to La Flor (JAC 26843, MX354). *Guanajuato*: Sierra Santa Rosa (RWB 07216, MX152); km 25, Hwy 57 between La Saucedá and San Miguel de Allende (AMNO 1337, MX250); Cerro Colorado

(KK HAR08-3, [MX362](#)). *Hidalgo*: 3 km N Epazoyucan (OFV 289, [MX 251](#)). *Jalisco*: approx. 30.5 km NE Lagos de Moreno on Hwy 80 (ANMO 1341, [MX252](#)); Vaquerias (UANL uncat., [MX151](#)); 10 mi (by road) S of El Platanar, just north of La Esperanza (UANL uncat., [MX154](#)); Tapalpa (JAC 26922, [MX356](#)). *Michoacán*: road to Quitupan south of Hwy 110 (RWB 0793, [MX 253](#)); Cuenca Rio Chiquito, Morelia (AQM Ha11, [MX153](#); AQM Ha24, [MX176](#)); Hwy 14 W Uruapan (JAC 26960, [MX357](#)); ca. 7 km S Tzitzio on Hwy 51 (JAC 27605, [MX359](#)). *Morelos*: Puente de Ixtla (JAC 27496, [MX358](#)). *Oaxaca*: Corral de Piedra, SE Huajuapán de León (KK HAR08-2, [MX348](#)). *Puebla*: Ejido Maravillas, Mpo. Teotlalco (CDR 135, [MX254](#)); Caltepec (KK HAR08-1, [MX349](#)); Zapotitlán de Salinas, Tilapa (MZFC 13918, [MX255](#)). *Querétaro*: Zamorano (FMQ 4160, [MX156](#)); Pueblo Nuevo, approx. 2 km E Cadereyta de Montes (MZFC 9245, [MX256](#)); Chiteje de la Cruz, Mpo. Amealco (MZFC 9365, [MX257](#)). *San Luis Potosí*: 2 km W Álvarez (MZFC 11078, [MX258](#)); Colonia Insurgentes, ca. 2.5 km W San Luis Potosí on Hwy 80 (MZFC 11215, [MX259](#)). *Sonora*: S Yécora (RWB 07359, [MX149](#)); approx. 1 km N Bacoachi on Hwy 89 (MZFC 15177, [MX260](#)). *Zacatecas*: N Ejido Mimbres, ca. 15 mi N Valpariaso (RWB 06121, [MX261](#)); Sierra de Organos (FMQ 4238, [MX155](#)); Hwy 54 just N Tabasco (JAC 26899, [MX355](#)). UNITED STATES: *Arizona*: Cochise Co: Price Canyon, Chiricahua Mountains. (PB 123/24, [MX178](#)). Coconino Co: Pumphouse Wash (PB 11/68, [MX159](#)); Geronimo Spring (PB 75/12, [MX292](#); PB 77/12, [MX370](#)); East Clear Creek (PB 96/18, [MX384](#)); Shinumu Creek, Grand Canyon (EMO GC0138, [MX387](#); EMO GC0141, [MX388](#)); Diamond Creek, Grand Canyon (KK TC71, [MX389](#); KK TC72, [MX390](#)). Gila Co: Houston Creek (PB 94/17, [MX330](#)). Graham Co: Tripp Canyon, Pinaleno Mountains (PB 28/3, [MX293](#)). Pima Co: Molino Canyon, Santa Catalina Mountains (PB 110/21, [MX368](#)). Santa Cruz Co: Madera Canyon, Santa Rita Mountains (PB 51/8, [MX177](#)); Gardner Canyon, Santa Rita Mountains (PB 39/5, [MX294](#)); Harshaw Canyon, Patagonia Mountains (PB 42/7, [MX371](#)). *Colorado*: Mesa Co: John Brown Canyon (KEK 153, [MX414](#)). *New Mexico*: Catron Co: Pueblo Creek, San Francisco

Mountains (PB 132/28, [MX378](#)). Grant Co: Mogollon Creek, Pinos Altos Mountains (PB 27/130, [MX158](#)); Gallinas Creek, Black Mountains (PB 125/25, [MX376](#)). *Hidalgo* Co: Coronado National Forest, W Cloverdale (RWB uncat., [MX329](#)). *Texas*: Jeff Davis Co: Musquiz Canyon, Hwy 118 (RWB uncat., [MX223](#)). *Utah*: Kane Co: Oak Creek, Zion (KK HAR130, [MX347](#)); Coyote Gulch (KEK 148, [MX415](#)).

HYLA EUPHORBIACEA

MEXICO: *Oaxaca*: Llano de las Flores (RWB 07107, [MX144](#)).

HYLA EXIMIA

MEXICO: *San Luis Potosí*: Las Lagunas, Mpo. Guadalcázar (RWB 06234, [MX37](#)). *Jalisco*: Tapalpa (UANL uncat., [MX40](#)). *Michoacán*: Morelia (AQM uncat., [MX65](#)). *Guanajuato*: Sierra Santa Rosa (RWB 07209, [MX128](#)). *Aguascalientes*: Sierra Fría (RWB 06118, [MX171](#)).

HYLA PLICATA

MEXICO: *Estado de México*: Los Tachos (RWB 0681, [MX33](#)).

HYLA WRIGHTORUM

MEXICO: *Chihuahua*: near Guachoci (RWB 07295, [MX125](#)); Colonia García (FMQ 4303, [MX341](#)). *Durango*: SE Mesa de las Navar (RWB 07304, [MX126](#)). USA: *Arizona*: Cochise Co: Scotia Canyon (RWB 07379, [MX122](#)). Coconino Co: near Bellemont (RWB 07330, [MX121](#)). *New Mexico*: Grant Co: Mogollon Mountains (JJ uncat., [MX123](#)).

OUTGROUPS: *Hyla andersonii* (AMCC 125627), *Hyla annectans* (CAS 215021), *Hyla arborea* (SAS 214), *Hyla avivoca* (LSU H-20811), *Hyla chinensis* (HL1), *Hyla chrysocelis* (LSU H-20900), *Hyla cinerea* (LSU H-19830), *Hyla femoralis* (LSU H-17966), *Hyla gratiosa* (LSU H-19067), *Hyla japonica* (LSU H-407), *Hyla meridionalis* (MVZ 177960), *Hyla savignyi* (MVZ 245918), *Hyla squirella* (LSU H-18304), *Hyla versicolor* (LSU H-2531), *Plectrohyla arborescens* (MVZ 231847), *Smilisca baudinii* (AMCC 118262).

Supporting Information

The following supporting information is available for this article:

Table S1. Details on individual haplotypes found within each POMC network.

Table S2. Details on individual haplotypes found within each cryB network.

Supporting Information may be found in the online version of this article.

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