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The role of mitochondrial introgression in illuminating the evolutionary history of Nearctic treefrogs

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Inferring the evolutionary history of lineages often becomes difficult when gene histories are in conflict with each other. Introgression, for example, can cause DNA sequences from one species to be more similar to sequences of a different species and lead to incongruence amongst gene trees. However, incorporating congruent and incongruent locus-specific phylogenetic estimates with the geographical distribution of lineages may provide valuable insight into evolutionary processes important to speciation. In this study, we investigated mitochondrial introgression within the *Hyla eximia* group to better understand its role in illuminating the evolutionary history and phylogeography of these treefrogs. We reconstructed and compared the matrilineal history of the *Hyla eximia* group with estimates of evolutionary history inferred from nuclear genes. We tested for introgression within the mitochondrial and nuclear genes using a posterior predictive checking approach. Reconstructions of the species tree based on the mitochondrial DNA (mtDNA) and nuclear DNA data were strongly discordant. Introgression between lineages was widespread in the mtDNA data set (145 occurrences amongst 11 of the 16 lineages), but uncommon in the nuclear genes (12 occurrences amongst four of the 16 lineages). Nonetheless, the geographical structuring of mtDNA within species provides valuable information on biogeographical areas, ancient areas of hybridization, and unique histories of lineages within the *H. eximia* group. These results suggest that the combination of nuclear, mitochondrial, and spatial information can provide a more complete picture of 'how evolutionary history played out', particularly in cases where mitochondrial introgression is known to occur.

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ADDITIONAL KEYWORDS: Hylidae – Mexico – mitochondrial capture – phylogeography – speciation.

INTRODUCTION

Coupling processes of diversification with spatial patterns of genetic structure is a central goal of phylogeographical research. However, achieving this goal can be complicated when gene histories are in conflict with each other. Two common reasons for genealogical discordance amongst loci are introgression and incomplete lineage sorting (Moore, 1995; Funk & Omland, 2003). Recent methodological advances using coalescent-based models can incorporate stochastic lineage sorting into phylogenetic estimation (Carstens & Knowles, 2007; Edwards, Liu & Pearl, 2007; Heled & Drummond, 2010). However, introgression, particularly deep in time, remains a confounding factor in phylogeographical research (Joly, McLenachan & Lockhart, 2009).

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Introgression involves the integration of DNA from one species into another species through the backcrossing of hybrids with pure parental types of one or both species. Introgression can cause DNA sequences from one species to be more similar to sequences of a different species and lead to incongruence amongst gene trees. Introgression can be relatively easy to distinguish after recent hybridization, which results in nonmonophyletic species in gene trees and shared sequences in areas of sympatry (Joly *et al*., 2009). Ancient introgression, however, may be more difficult to detect. As time since hybridization increases, introgressed sequences are more likely to sort and the geographical signals of introgression are more likely to erode (Funk & Omland, 2003; Linnen & Farrell, 2007). Identifying ancient introgression events becomes challenging because genetic lineages will have become reciprocally monophyletic in formerly hybridizing species and direct evidence for donor taxa is erased (Linnen & Farrell, 2007).

The maternally inherited mitochondrial genome often introgresses more rapidly than biparentally or paternally inherited components of the nuclear genome (Ballard & Whitlock, 2004; Chan & Levin, 2005; but see Haldane's rule, e.g. Carling & Brumfield, 2008). The smaller effective population size of mitochondrial DNA (mtDNA) compared with nuclear loci may facilitate this process, such that even low levels of introgression may be sufficient to establish an introgressed mitochondrial haplotype in a foreign population (Takahata & Slatkin, 1984; Ballard & Whitlock, 2004; Chan & Levin, 2005). This process can lead to the complete replacement of the mtDNA in the recipient species by mitochondrial genome capture (e.g. Rabosky *et al*., 2009; Reid, Demboski & Sullivan, 2012; Tang *et al*., 2012; Willis, Farias & Ortí, 2014). Rapid genetic drift in small populations and adaptive introgression can both accelerate the rate of mitochondrial capture (Ballard & Whitlock, 2004; Toews & Brelsford, 2012).

Ancient hybridizations and mitochondrial gene capture followed by reproductive isolation may result in the establishment of a new evolutionary trajectory in the introgressed maternal genome over time and the development of new lineage-specific mutations (Bryson *et al*., 2010). As a result, the geographical distribution of lineages with a captured mtDNA genome may also be informative. For example, the horned lizards *Phrynosoma platyrhinos* and *Phrynosoma goodei* appear to have the captured mitochondrial genome of *Phrynosoma mcalli* (Leaché & McGuire, 2006), yet several studies inferred phylogeographical patterns in these species using only mtDNA (Jones, 1995; Mulcahy *et al*., 2006; Luxbacher & Knouft, 2009; Jezkova, 2010). If hybridization and mtDNA capture was followed by reproductive isolation, then mutations in the mtDNA of *P. platyrhinos* and *P. goodei* should develop independent of *P. mcalli*. Through time this would result in genetic structuring in the mitochondrial genomes of *P. platyrhinos* and *P. goodei* that reflect their evolutionary history postdating introgression. Indeed, marked genetic structuring in the mtDNA of *P. platyrhinos* and *P. goodei* corresponds well to historical processes across the distribution of these lizards (Mulcahy *et al*., 2006). Phylogeographical inference from introgressed mtDNA has provided similar insight into the evolutionary histories of a number of other taxa, including fish (Willis *et al*., 2007; Gross, 2012), mammals (Shields *et al*., 2000; Good *et al*., 2008), and invertebrates (Zakharov *et al*., 2009; Marková *et al*., 2013).

Mitochondrial introgression and gene capture have been documented within Nearctic hylid treefrogs in the *Hyla eximia* group (Bryson *et al*., 2010; Klymus *et al*., 2010; Klymus & Gerhardt, 2012). The *H. eximia* group is broadly distributed across the moderate to high elevations of south-western North America (Fig. 1), ranging from southern Utah and Colorado south across most of mainland Mexico and into Guatemala (Duellman, 2001). The taxonomic composition of this group has changed several times over the past 50 years (Duellman, 1970, 2001; Hua *et al*., 2009), but the core group is comprised of *Hyla arenicolor*, an undescribed species from the Balsas Basin of Mexico (Bryson *et al*., 2010; Klymus & Gerhardt, 2012), and the morphologically and ecologically similar species *Hyla arboricola*, *Hyla euphorbiacea*, *Hyla eximia*, *Hyla plicata*, *Hyla walkeri*, and *Hyla wrightorum* (Eliosa-León, 2002a, b; Smith *et al*., 2007; Hua *et al*., 2009; Bryson *et al*., 2010; Klymus & Gerhardt, 2012). Although *H. arenicolor* and the Balsas Basin species occupy rocky canyons and streams, the remaining species inhabit similar highland habitats and are generally associated with mixed pine–oak forests.

In this study, we expanded the investigation of mtDNA introgression within the *H. eximia* group across a larger geography, more populations, and more species to better understand its role in revealing the evolutionary history and phylogeography of these treefrogs. We expected that the introgression of a mitochondrial genome into another species should not only result in mitochondrial/nuclear DNA discordance but also restart the progression of mtDNA evolutionary divergence from that moment onward. As a result, we predict that incorporating congruent and incongruent locus-specific phylogenetic estimates with the geographical distribution of lineages will provide valuable insight into evolutionary history. We tested this expectation by reconstructing and comparing the matrilineal phylogeny of the *H. eximia* group with the phylogenetic history inferred from nuclear genes. We confirmed introgression between lineages using a posterior predictive checking model and data from both the mtDNA and nuclear DNA (nuDNA).

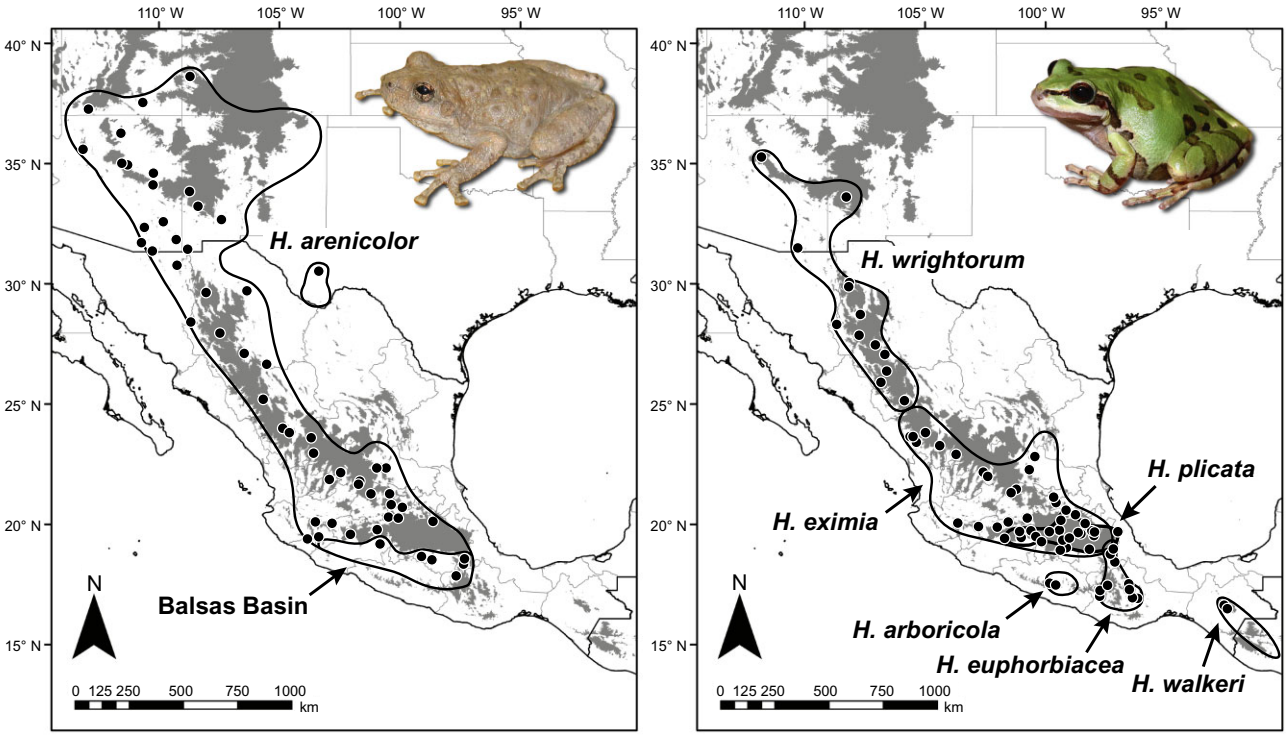


Figure 1. Distribution of *Hyla eximia* group treefrogs (thick black lines). Black dots indicate localities sampled in this study (listed in Table S1). The Balsas Basin lineage represents an undescribed species within the group (Bryson *et al*., 2010; Klymus & Gerhardt, 2012).

MATERIAL AND METHODS

Taxon sampling and laboratory methods

We collected tissue samples from 128 frogs of the currently recognized species in the *H. eximia* group from throughout their distributions (Fig. 1; Table S1). We also included samples from *H. arboricola*, recently removed from the synonymy of *H. eximia* (Eliosa-León, 2002a, b). We did not include samples of *Hyla bocourti*, a possible member of the *H. eximia* group (Duellman, 2001) that may be extinct (Acevedo & Young, 2004). We used *Hyla cinerea* and *Hyla versicolor* as outgroups (Pyron & Wiens, 2011). We augmented sampling with tissues obtained from museum collections, indicated in Table S1.

Following methods specified in Bryson *et al*. (2010), we extracted genomic DNA from toe or liver tissue and sequenced three mitochondrial DNA gene regions, including part of the *NADH dehydrogenase subunit 4* and its flanking tRNAs (*ND4*, 759 bp) and the complete *ATPase subunits 8* and *6* (*ATPase8*, 165 bp; *ATPase6*, 684 bp; with an overlap of 10 bp between the two genes). We also sequenced three nuclear genes, including 520 bp of *proopiomelanocortin A* (*POMC*), 169 bp of *β-crystallin* (*cryB*), and 517 bp of *tyrosinase* (*Tyr1*). Primer sequences for *Tyr1* were from Bossuyt & Milinkovitch (2000). We edited and manually aligned forward and reverse sequences for each individual using SEQUENCHER v. 4.2 (Gene Codes Corporation, Ann Arbor, MI). For *cryB* data, which contained numerous indels, we performed an additional sequence alignment with MAFFT v. 6 (Katoh *et al*., 2002; Katoh & Toh, 2008) using default settings and the G-INS-i algorithm. We computationally determined the gametic phase of heterozygous variants using PHASE v. 2.1.1 (Stephens & Donnelly, 2003). For each nuDNA data set, separate runs of 400 iterations each were carried out, accepting results with a probability threshold of 0.7 or higher. All polymorphic sites with a probability less than 0.70 were coded in both alleles with the appropriate IUPAC ambiguity code. To test for recombination within nuclear loci, we performed three independent recombination tests (RDP, GENECOV, and MaxChi) in the program RDP v. 3.44 (Martin *et al*., 2010) using the default settings for all three methods.

Phylogeographical estimation

We generated a mtDNA phylogeny using MrBayes v. 3.1 (Ronquist & Huelsenbeck, 2003) to examine geographical structure within the *H. eximia* group. Geographical lineages within *H. arenicolor* inferred in a previous study from either mtDNA or amplified fragment length polymorphism data were nearly

identical (Klymus & Gerhardt, 2012), which suggests that mtDNA is a useful marker for broadly delimiting geographical lineages within the *H. eximia* group. We used MrModeltest v. 2.1 (Nylander, 2004) to select the best-fit model of evolution for each gene region, based on Akaike information criteria. Analyses in MrBayes consisted of four runs (nruns = 4) conducted with three heated and one cold Markov chain sampling every 100 generations for 5 000 000 generations. We used a mean branch length exponential prior of 100 and heating temperature of 0.02, which improved mixing and convergence. We verified that the average standard deviation of split frequencies was below 0.01 at the end of the analysis and visualized output parameters using TRACER v. 1.4 (Rambaut & Drummond, 2007) to further ascertain convergence and stationarity. We discarded all samples obtained during the first 1 000 000 (25%) generations as burn-in.

We used a general mixed Yule coalescent model (GMYC) to delineate geographically distinct clusters of samples (hereafter referred to as 'lineages'). The GMYC model identifies independent evolutionary clusters by detecting a threshold value at the transition from interspecific to intraspecific branching patterns. Although this model has been recently criticized for potentially over-splitting species with pronounced genetic structure (Satler, Carstens & Hedin, 2013), it remains an effective way to estimate genetically distinct geographical clusters at the phylogeographical (intraspecific) scale. We used SPLITS (Pons *et al*., 2006) in the R command-line package (R Development Core Team, 2011) to run a likelihood implementation of the GMYC model. We performed both single and multiple threshold versions of the model. The multiple threshold variant tests whether allowing the species-coalescent transition to vary across the tree significantly improves the fit to the model (Monaghan *et al*., 2009).

We generated an ultrametric tree for use in SPLITS using BEAST v. 1.7.2 (Drummond & Rambaut, 2007). We used separate models of evolution for each of the four gene regions and we ran the analyses for 40 000 000 generations, with samples retained every 1000 generations, using a Yule tree prior. Results were displayed in TRACER to confirm acceptable mixing and likelihood stationarity, appropriate burn-in, and adequate effective sample sizes above 200 for all estimated parameters. After discarding the first 4 000 000 generations (10%) as burn-in, the parameter values of the samples from the posterior distribution were summarized on the maximum clade credibility tree using TreeAnnotator v. 1.7.2 (Drummond & Rambaut, 2007).

Species trees and divergence times

We used *BEAST (Heled & Drummond, 2010), a part of the BEAST v. 1.6.2 package (Drummond & Rambaut, 2007), to estimate time-calibrated species trees. Because the mtDNA data were suspected to support a different topology as a result of mitochondrial introgression and gene capture (Bryson *et al*., 2010; Klymus *et al*., 2010; Klymus & Gerhardt, 2012), we analysed the mtDNA and nuDNA data sets separately. In our mtDNA species-tree reconstructions, we included all samples ($N = 123$) and the two outgroups with the exception of samples of *H. arenicolor* from the Grand Canyon of Arizona, which contain introgressed mtDNA indistinguishable from *H. wrightorum* (Bryson *et al*., 2010; Klymus *et al*., 2010; Klymus & Gerhardt, 2012). In our nuDNA species-tree analyses, we included two to four individuals ($N = 35$) from each phylogeographical lineage inferred from our mtDNA data and the two outgroups. We estimated best-fit models of evolution for each gene using MrModeltest. In each species-tree analysis we used relaxed uncorrelated lognormal clocks for each gene and a Yule speciation prior. For mtDNA analyses, we unlinked substitution and clock models for each gene region. We ran analyses for 100 000 000 generations, with samples retained every 1000 generations. Results were displayed in TRACER to confirm acceptable mixing and likelihood stationarity, appropriate burn-in, and adequate effective sample sizes. After discarding the first 10% of generations as burn-in, the parameter values of the samples from the posterior distribution were summarized on the maximum clade credibility tree using TreeAnnotator. We repeated this burn-in and visualization procedure for each of the three nuclear gene trees coestimated by *BEAST as part of the nuDNA species-tree reconstruction.

To calibrate our mtDNA species tree, we used the mean rate estimated from the fossil-calibrated chronogram of *Hyla* in Bryson *et al*. (2010). This mean rate, estimated for the same mtDNA genes used here, was 9.63×10^{-3} substitutions/site/million years (1.926% change between lineages per million years). We gave the mtDNA rate a lognormal distribution and specified a logarithmic standard deviation of 0.2 around this mean to produce a wide upper and lower 95% prior credibility interval of 6.79×10^{-3} and 1.312×10^{-2}, similar to the 95% posterior credibility interval in Bryson *et al*. (2010).

To calibrate our nuDNA species tree, we estimated clock rates relative to a mtDNA rate. Using MEGA5 (Tamura *et al*., 2011), we calculated mean between-group uncorrected *p*-distances from mtDNA data and each of the three nuclear genes for three paired groupings in our study: *H. arenicolor* from the Sonoran Desert/*H. arenicolor* from the Chihuahuan Desert + Central Mexican Plateau, *H. plicata*/*H. euphorbiacea*, and the Balsas Basin species/*H. eximia* from the southern Sierra Madre Occidental + Central Mexican Plateau. For each nuclear gene, genetic distances between each paired grouping were divided by the corresponding mtDNA

distances, and the average of these three numbers calculated to obtain a comparative mutation rate relative to mtDNA. These relative rates were multiplied by the mtDNA mutation rate from above (9.63×10^{-3}). Using this method, we obtained the following rates for substitutions/site/million years: *cryB* = 4.43×10^{-3}, *POMC* = 2.99×10^{-3}, and *Tyr1* = 1.35×10^{-3}. The calculated rate for the *Tyr1* gene was similar to the *Tyr1* rate estimated for the Hyloidae superfamily of frogs (which includes *Hyla*) using fossil data (1.95×10^{-3}, Fouquet *et al*., 2012). This similarity is important because it suggests that our divergence dates may still be informative despite a history of introgression in the *H. eximia* group (see below). In fact, reconstructing the nuclear species tree with the *Tyr1* substitution rate from Fouquet *et al*. (2012), with rates for *cryB* and *POMC* estimated relative to *Tyr1* by *BEAST, yields generally similar divergence dates across the tree (e.g. dates are within 2 000 000 years of each other at the base of the tree, within 1 000 000 years at the base of the *H. eximia* group, and identical at the base of the *H. arenicolor* clade). As with our mtDNA species-tree analyses, we used a lognormal distribution and a relatively wide logarithmic standard deviation of 0.2 for the prior distribution of each nuclear gene's mutation rate.

Introgression

Although previous studies (Bryson *et al*., 2010; Klymus *et al*., 2010; Klymus & Gerhardt, 2012) have suggested mtDNA introgression between *H. arenicolor* and other species of the *H. eximia* group, this hypothesis has not been formally tested using a statistical framework. Further, limited evidence suggests that nuclear introgression between *H. arenicolor* and the *H. eximia* group might also be possible (Bryson *et al*., 2010). As our species-tree analyses assume that the only process creating discordance amongst loci is genetic drift within species (Liu & Pearl, 2007), incorporating gene trees that have topologies resulting from introgression could alter the parameter estimates for the model and skew the species-tree topology (Willis *et al*., 2014).

We used a posterior predictive checking approach (Joly *et al*., 2009) implemented in JML v. 1.01 (Joly, 2012) to test for introgression between lineages for all loci. This program tests whether the minimum genetic distance between the sequences of two individuals is smaller than those simulated under a model involving no hybridization such as incomplete lineage sorting (Joly *et al*., 2009). JML uses as input the posterior of species trees with branch lengths and population sizes from *BEAST to generate a gene tree from which DNA sequences are then simulated. A test quantity, the minimum pairwise distance between sequences of two species, is estimated from the observed and simulated data sets to determine how well the model fits the data. If the observed pairwise distance is smaller than 95% of the simulated value, then a model involving no hybridization can be rejected and it can be concluded that the inaccuracy of the model is because of the presence of hybrid sequences (Joly *et al*., 2009).

We conducted two separate analyses. First, we inferred a species tree in *BEAST using both mtDNA and nuDNA and the same priors as specified above. We used the posterior distribution of species trees, population sizes, and branch lengths to simulate gene trees and sequence data sets in JML under the coalescent with no migration. We then assessed whether introgression could be detected for any pair of individuals in any locus at a probability of < 0.05. However, this species-tree estimate may be skewed as a result of conflict between the mtDNA and nuclear gene trees owing to mtDNA introgression (e.g. Eckert & Carstens, 2008). Accordingly, we repeated the analysis using a species tree reconstructed in *BEAST using only the nuDNA, and followed the assumption that this second analysis should result in a more accurate estimate of introgression within the nuclear loci.

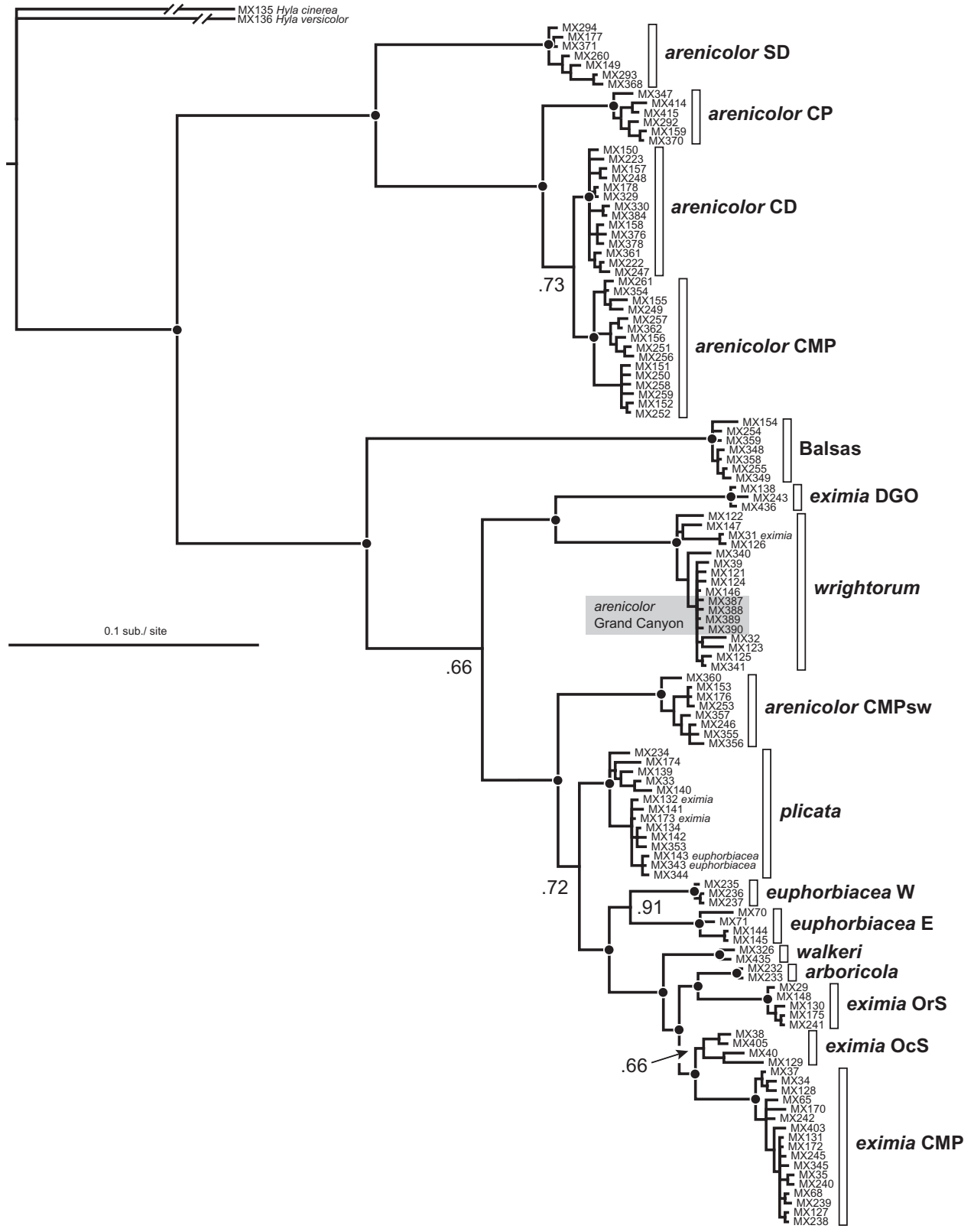
RESULTS

Genetic data

The full mtDNA data set (1598 bp) contained 482 parsimony-informative sites. The nuclear loci contained less variation than the mtDNA (parsimony-informative sites, *POMC*: 76/520 bp; *cryB*: 45/169 bp; *Tyr1*: 63/517 bp). We were unable to obtain ATPase sequence data for two individuals (MX435 *H. walkeri* and MX436 *H. eximia*). Models of sequence evolution selected for gene partitions were GTR + I + G (*ND4*, *ATPase6*) and HKY + G (*ATPase8*, tRNAs) in the mtDNA gene tree data set, and GTR + I + G (*ND4*, *ATPase6*, *POMC*), HKY + G (*ATPase8*, tRNAs), GTR + I (*cryB*), and HKY + I (*Tyr1*) for the species-tree data sets. None of the three methods used in RDP detected recombination in the nuclear genes. All aligned sequences were deposited in the Dryad repository: http://dx.doi.org/10.5061/dryad.3rc02.

Phylogeographical estimation

We identified two major mtDNA clades within the *H. eximia* group (Fig. 2). One clade contained most of the *H. arenicolor* samples. The second clade contained the Balsas Basin species, *H. arenicolor* from the south-western region of the Central Mexican Plateau, *H. arenicolor* from the Grand Canyon of Arizona, and the six morphologically and ecologically similar species *H. eximia*, *H. wrightorum*, *H. plicata*, *H. euphorbiacea*, *H. arboricola*, and *H. walkeri*.


MX135 Hyla cinerea
MX136 Hyla versicolor
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MX233
arboricola
MX29
MX148
MX130
MX175
MX241
eximia OrS
MX38
MX405
MX40
MX129
eximia OcS
.66
MX37
MX34
MX128
MX65
MX170
MX242
MX403
MX131
MX172
MX245
MX345
MX35
MX240
MX68
MX239
MX127
MX238
eximia CMP

Figure 2. Phylogeographical structure within *Hyla eximia* group treefrogs inferred from Bayesian analyses of 1598 bp of mitochondrial DNA. Lineages were inferred using a single-threshold general mixed Yule coalescent model. All major nodes that received ≥ 0.95 Bayesian posterior probability are depicted with black dots. Bayesian posterior probability support values at all other nodes are provided. Abbreviations: CD, Chihuahuan Desert; CMP, Central Mexican Plateau; CMPsw, south-western Central Mexican Plateau; CP, Colorado Plateau; DGO, Durango; E, eastern; OcS, southern Sierra Madre Occidental; OrS, southern Sierra Madre Oriental; SD, Sonoran Desert; W, western.

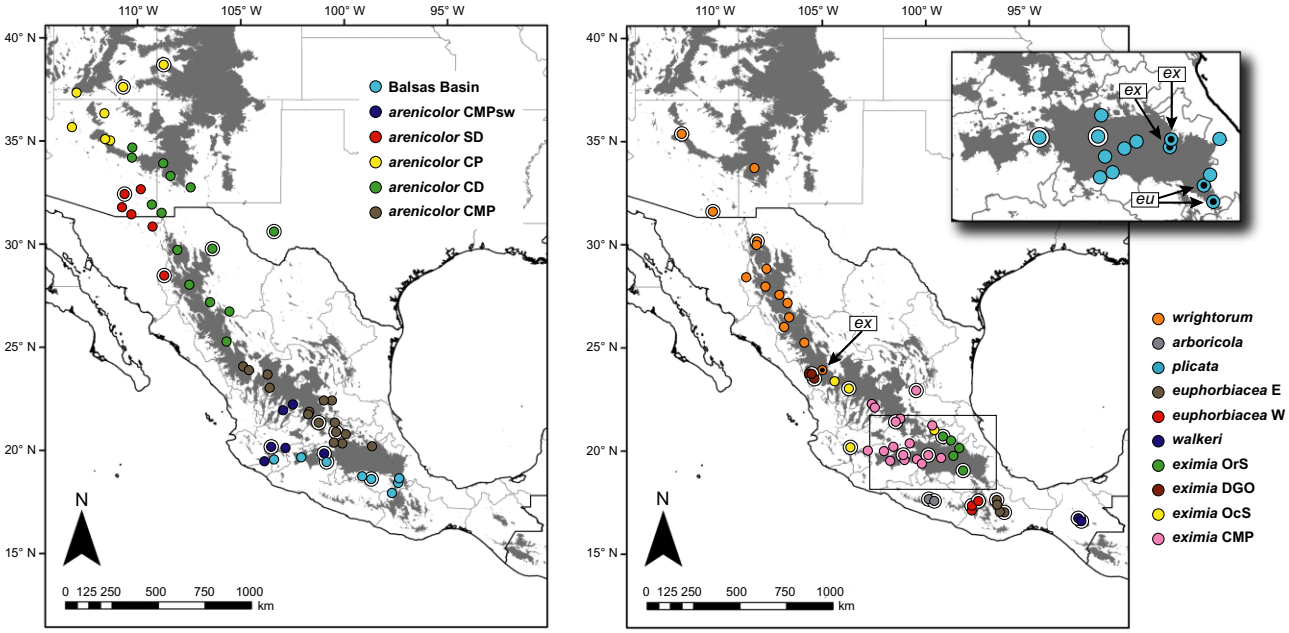

Figure 3. Geographical distribution of inferred mitochondrial lineages within *Hyla eximia* group treefrogs. Lineages are colour-coded to correspond to haplotypes plotted on the map. Circled dots indicate localities of samples used in multilocus species-tree reconstructions. Five mismatches between species designation (*H. eximia*, abbreviated *ex*; *Hyla euphorbiacea*, abbreviated *eu*) and mitochondrial lineage are noted. Abbreviations: CD, Chihuahuan Desert; CMP, Central Mexican Plateau; CMPsw, south-western Central Mexican Plateau; CP, Colorado Plateau; DGO, Durango; E, eastern; OcS, southern Sierra Madre Occidental; OrS, southern Sierra Madre Oriental; SD, Sonoran Desert; W, western.

The single and multiple threshold GMYC models were not significantly different from each other ($X^2 = 6.315$, df = 3, $P = 0.097$) so results from the single threshold model were used to infer phylogeographical lineages. The 16 independent evolutionary clusters estimated by the GMYC model were generally concordant with taxonomy and geography (Figs 2, 3). *Hyla wrightorum*, *H. plicata*, *H. walkeri*, and *H. arboricola* each formed single lineages. One haplotype of *H. eximia* (MX31) from Durango was nested within the *H. wrightorum* lineage (Fig. 2). Two haplotypes of *H. euphorbiacea* and two haplotypes of *H. eximia* grouped with *H. plicata* haplotypes within the *H. plicata* lineage (Fig. 2). All haplotypes of *H. arenicolor* from the Grand Canyon of Arizona grouped with *H. wrightorum*, consistent with previous studies (Klymus *et al.*, 2010; Klymus & Gerhardt, 2012). Pronounced genetic structure was detected in *H. arenicolor*, *H. eximia*, and *H. euphorbiacea*, and each species contained several distinct geographically delimited lineages (Figs 2, 3). Five lineages of *H. arenicolor* were identified from well-defined biogeographical regions, including the Sonoran Desert and associated highlands (*arenicolor* SD), Chihuahuan Desert and associated highlands (*arenicolor* CD), Colorado Plateau (*arenicolor* CP), Central Mexican Plateau (*arenicolor* CMP), and south-western region of the Central Mexican Plateau (*arenicolor* CMPsw). Haplotypes of *H. arenicolor* from the Grand Canyon of Arizona were indistinguishable from *H. wrightorum*. *Hyla eximia* formed four lineages distributed in south-western Durango (*eximia* DGO), the southern Sierra Madre Oriental (*eximia* OrS), across the Central Mexican Plateau (*eximia* CMP), and in the southernmost Sierra Madre Occidental and two sites to the south (Tapalpa, Jalisco) and south-east (Nuevo San Joaquin, Querétaro; *eximia* OcS). This Nuevo San

Joaquin, Querétaro locality was well within the distribution of the Central Mexican Plateau lineage (Fig. 3); otherwise, the distributions of the four lineages of *H. eximia* were non-overlapping. The two lineages of *H. euphorbiacea* corresponded to regions of the Sierra Madre del Sur in eastern (*euphorbiacea* E) and western (*euphorbiacea* W) Oaxaca.

Species trees and divergence times

Reconstructions of the species tree based on the mtDNA and nuDNA data were strongly discordant (Fig. 4). Phylogenetic relationships amongst the lineages varied between the two species trees to such an extent that relationships between only four of the 16 lineages were similar in both trees (the sister relationship between *H. arenicolor* from the Chihuahuan Desert and Central Mexican Plateau, and the sister relationship between *H. eximia* from the southern Sierra Madre Occidental and the Central Mexican Plateau). Although nodal support across both species trees was generally weak, even the well-supported nodes in each tree supported different relationships. Estimated divergence dates in the mtDNA species tree were noticeably older and 95% posterior credibility intervals considerably wider compared with the nuDNA species tree. For example, the basal divergence within the *H. eximia* group was estimated to have occurred 15.8 Mya (24.3–8.0 Mya) in the mtDNA species tree. However, the estimated date of this divergence event in the nuDNA species tree was many millions of years later, around 6.4 Mya (8.8–4.2 Mya). Based on mean estimated dates of divergence, 47% of the divergences within the *H. eximia* group in the mtDNA species tree occurred during the Pleistocene as compared to 67% in the nuDNA species tree. Each of the three nuclear gene trees contained limited phylogenetic resolution and relatively few strongly supported nodes, especially towards the base of the trees (Fig. S1). However, samples of several lineages consistently grouped together, such as *H. walkeri*, *H. wrightorum*, and Balsas Basin. Lineages of *H. arenicolor* formed a monophyletic group in the *Tyr1* and *cryB* gene trees, and samples of *H. arboricola* formed a monophyletic group in the *POMC* and *cryB* gene trees.

Introgression

Results from JML suggested that introgression between lineages was widespread in the mtDNA data set. The analysis detected 145 instances of potential introgression involving samples from all lineages except the Balsas Basin and *H. arenicolor* from the Sonoran and Chihuahuan Deserts, Colorado Plateau, and Central Mexican Plateau (Table S2). The nuDNA data set, however, contained relatively few occurrences of potential introgression. Based on analyses using the species tree reconstructed from both mtDNA and nuDNA, there was no strong evidence for introgression in the *POMC* or *Tyr1* data sets. Introgression in the *cryB* data set was detected between *H. arboricola* and *H. euphorbiacea* sequences. JML results inferred from the species tree estimated using only the nuDNA suggested no significant introgression in the *Tyr1* and *cryB* and only four occurrences in the *POMC* data set (involving one sequence of *H. eximia* from the southern Sierra Madre Oriental and one sequence of *H. plicata*, and one sequence of *H. eximia* from the southern Sierra Madre Oriental and three sequences of *H. arboricola*).

DISCUSSION

mtDNA introgression in the *H. eximia* group

Although previous studies have provided evidence for mitochondrial introgression and gene capture between *H. arenicolor* and *H. eximia*, and *H. arenicolor* and *H. wrightorum* (Bryson *et al*., 2010; Klymus *et al*., 2010; Klymus & Gerhardt, 2012), results from our study suggest that these phenomena are widespread amongst nearly all species in the *H. eximia* group. Within the six morphologically and ecologically similar species *H. eximia*, *H. wrightorum*, *H. plicata*, *H. euphorbiacea*, *H. arboricola*, and *H. walkeri*, two lineages in particular appear to have captured the mtDNA genome of a different lineage. Three *H. eximia* from three different localities in south-western Durango form a genetically distinct monophyletic lineage that is strongly supported (1.0 posterior probability, PP) as sister to *H. wrightorum* based on analyses of mtDNA (Figs 2, 4). However, analyses of the nuDNA alternatively suggest that all lineages of *H. eximia* form a monophyletic group (0.91 PP), consistent with call and morphological data (Duellman, 2001). Two samples of *H. arboricola* from the Sierra Madre del Sur in Guerrero are placed as sister to *H. eximia* from the southern Sierra Madre Oriental in the mtDNA gene tree (1.0 PP, Fig. 2) and mtDNA species tree (0.72 PP, Fig. 4). However, the nuDNA species tree strongly supports (1.0 PP) the placement of *H. arboricola* in a clade with two geographically proximate treefrogs, *H. plicata* from the Trans-Mexican Volcanic Belt and *H. euphorbiacea* from the Sierra Madre del Sur (Figs 3, 4). The gene histories of the mtDNA and nuDNA in *H. eximia* group treefrogs clearly appear to be in conflict.

Evolutionary history of the *H. eximia* group

Our time-calibrated species tree reconstructed from nuDNA (Fig. 4) provides a reasonable estimate of the evolutionary history of the *H. eximia* group as introgression within the nuclear genes appears to be low. Unfortunately, many of the nodes across this tree are not well supported, perhaps because of the reduced

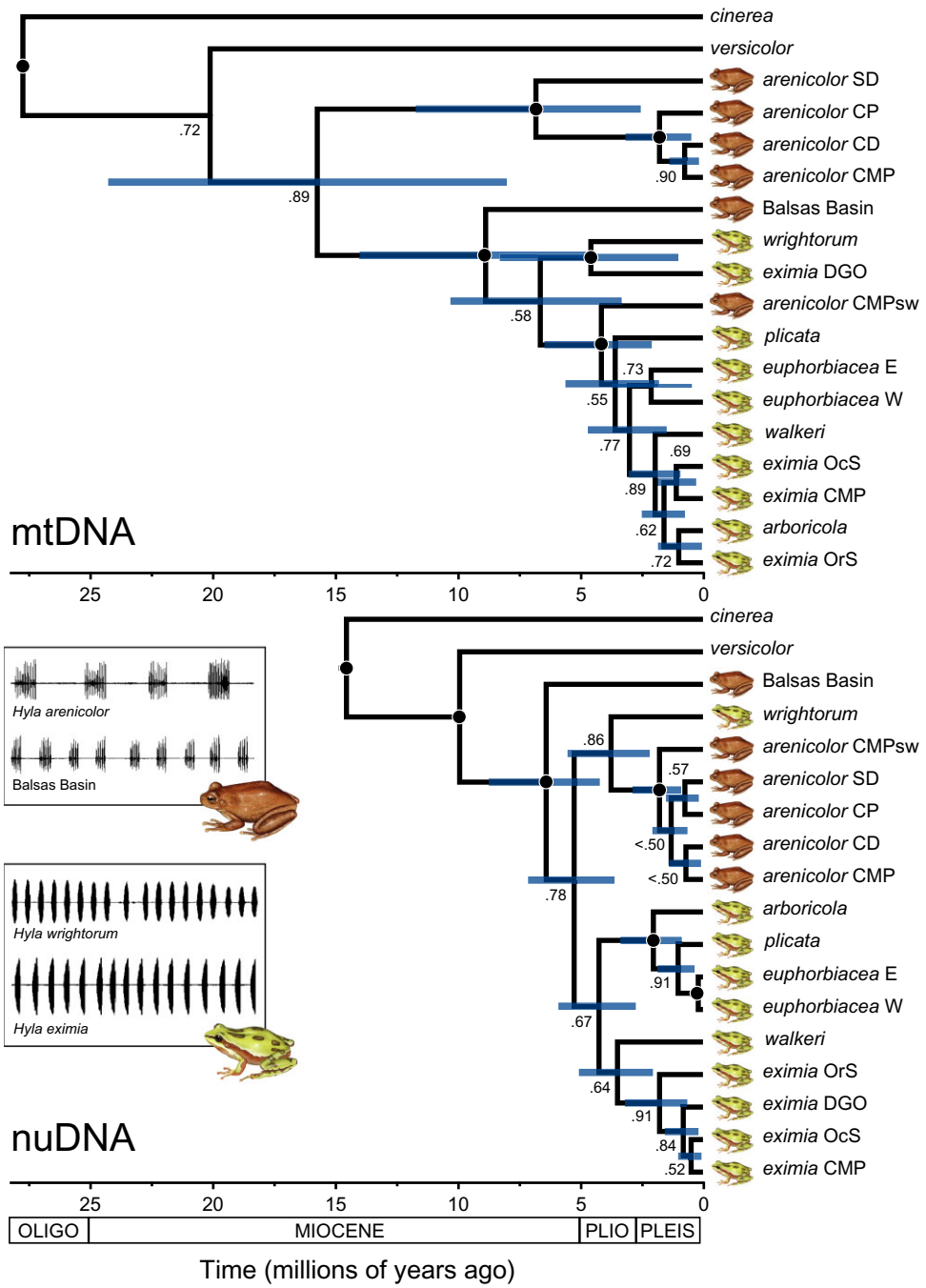

Figure 4. Species-tree reconstructions for *Hyla eximia* group treefrogs estimated from mitochondrial DNA (mtDNA) or nuclear DNA (nuDNA) using *BEAST. Bars indicate 95% posterior credibility intervals of divergence dates. All major nodes that received ≥ 0.95 Bayesian posterior probability are depicted with black dots. Bayesian posterior probability support values at all other nodes are provided. Inset shows oscillograms of the advertisement calls (90 s) of four *H. eximia* group species. Note call similarities between *Hyla wrightorum* and *H. eximia* and call differences between *H. wrightorum* and *Hyla arenicolor* (data from Klymus, Humfeld & Gerhardt, 2012). Abbreviations: CD, Chihuahuan Desert; CMP, Central Mexican Plateau; CMPsw, south-western Central Mexican Plateau; CP, Colorado Plateau; DGO, Durango; E, eastern; OcS, southern Sierra Madre Occidental; Oligo, Oligocene; OrS, southern Sierra Madre Oriental; Pleis, Pleistocene; Plio, Pliocene; SD, Sonoran Desert; W, western.

phylogenetic signal in the three nuclear genes (Fig. S1). As a result it is difficult to confidently discern the relationships of many lineages. Nonetheless, several patterns within the species tree are evident. First, three clades appear to be reasonably supported (> 0.90 PP): *H. eximia*; *H. arenicolor*; and *H. arboricola* + *H. euphorbiacea* + *H. plicata*. The compositions of the former two clades match current taxonomic designations based on mating calls and morphology (Duellman, 2001; Klymus *et al*., 2010; Klymus & Gerhardt, 2012). The latter clade is a composite of three species distributed in southern Mexico (Fig. 3) across three mountain ranges with shared biotic affinities (Paniagua & Morrone, 2009). Second, speciation within each of these three clades occurred nearly entirely within the Pleistocene. Estimated divergence dates between lineages of *H. arenicolor* are dramatically younger than those based on an mtDNA gene-tree estimate (Bryson *et al*., 2010). As a result, diversification in these and other *H. eximia* group treefrogs was probably more heavily impacted by Pleistocene glacial–interglacial cycles than older Neogene events. Lastly, the inclusion of *H. wrightorum* with *H. arenicolor* (0.86 PP) and the basal placement of the Balsas Basin lineage within the *H. eximia* group (0.78 PP) are both contrary to relationships based on call and morphology data (Klymus & Gerhardt, 2012, and references therein). These relationships suggest that either morphology and mating calls are plastic or that a more complex history of hybridization within the *H. eximia* group exists that was undetected by our sampling and JML analyses. Nearly identical mating calls and similar morphologies between *H. wrightorum* and *H. eximia* (Klymus & Gerhardt, 2012; Fig. 4), both inferred as nonsister species in our species tree, suggest that the latter explanation may be true. Further, the lack of significant evidence for hybridization inferred from our JML analyses simply suggests that the program could not distinguish whether any incongruence was the result of introgression or incomplete lineage sorting, which is not evidence that hybridization was completely absent (Joly *et al*., 2009). Future studies with more nuclear loci may reveal an additional layer of complexity within the evolutionary history of the *H. eximia* group.

Is the mtDNA genome in the *H. eximia* group still informative?

Although it seems clear that mtDNA introgression and gene capture severely limit the utility of this marker for reconstructing phylogenetic relationships within the *H. eximia* group, can mtDNA still provide insight into the evolutionary history of these widespread treefrogs? At a minimum, the general concordance between the geographical distribution of mitochondrial lineages and biogeographical regions suggests that mtDNA still retains important information about the phylogeographical structure of these treefrogs across south-western North America. Previous studies have demonstrated that at least four of the five maternal lineages of *H. arenicolor* and the Balsas Basin species inhabit distinct biogeographical areas (Barber, 1999; Bryson *et al*., 2010; Klymus *et al*., 2010; Klymus & Gerhardt, 2012), including the Sonoran and Chihuahuan Deserts, Colorado Plateau, Central Mexican Plateau, and Balsas Basin. One lineage of *H. arenicolor* is distributed across the south-western corner of the Central Mexican Plateau, a region also inhabited by a lineage of *H. eximia* (Fig. 3) and a lineage of Mexican gophersnake (Bryson, García-Vázquez & Riddle, 2011a). Most of the remaining nine lineages within the *H. eximia* group are also allopatrically distributed within distinct biogeographical regions (Fig. 3). *Hyla wrightorum* is distributed broadly across the Sierra Madre Occidental and adjacent northern highlands, *H. plicata* is distributed almost exclusively within the higher elevations of the Trans-Mexican Volcanic Belt, and *H. walkeri* is found along the Chiapan Highlands. *Hyla arboricola* occurs in the western Sierra Madre del Sur of Guerrero, a region characterized by high biotic endemism (Fa & Morales, 1993). Of the three remaining lineages of *H. eximia*, one inhabits the Central Mexican Plateau and one is distributed along the southern Sierra Madre Oriental, a highland region that contains other distinct phylogeographical lineages (Durish *et al*., 2004; Bryson *et al*., 2011b, c). The third lineage of *H. eximia* occurs in the humid pine–oak forests of south-western Durango. Although this area lies within the Sierra Madre Occidental, the wetter climate on the Pacific versant supports a humid pine–oak forest that differs from the drier, cooler pine–oak forests along the interior (Webb, 1984) and contains several narrow endemics (Tanner, Dixon & Harris, 1972; Bryson *et al*., 2007). Lastly, two lineages of *H. euphorbiacea* are distributed in regions of the Sierra Madre del Sur in eastern and western Oaxaca. Similar genetic lineages have been found in a number of highland taxa (Parra-Olea *et al*., 2005; Puebla-Olivares *et al*., 2008; Ruiz-Sanchez & Specht, 2013).

The mitochondrial genome of *H. eximia* group treefrogs may also point to ancient areas of hybridization and episodes of gene capture from extinct ancestors. For example, results from JML suggest that *H. arboricola* from the Sierra Madre del Sur in Guerrero and *H. eximia* from the southern Sierra Madre Oriental have a history of hybridization. However, these two areas are currently isolated from each other by the arid Balsas Basin drainage (Fig. 3). Hybridization provides evidence for a recent connection of these regions by ephemeral corridors of pine–oak woodlands during Pleistocene glacial periods (Bryson *et al*., 2011b). Similarly, *H. arenicolor* from the Grand Canyon of Arizona and

H. wrightorum from the pine forests of northern Arizona are geographically isolated yet share a similar mitochondrial genome, suggesting hybridization occurred in this region during the Pleistocene (Bryson *et al*., 2010). The deep mtDNA divergence of the Sonoran Desert lineage of *H. arenicolor* from other *H. arenicolor* (Figs 2, 4), a pattern not recovered with nuDNA (Fig. 4), provides evidence for a unique history of this lineage. One possible explanation is that the Sonoran Desert lineage possesses the introgressed mitochondrial genome of a divergent extinct lineage of *H. arenicolor* (Klymus & Gerhardt, 2012) and thus represents an 'mtDNA fossil' (Bossu & Near, 2009). This may also be the case for the Balsas Basin lineage. Such a scenario has been posited previously for hylid frogs (Holloway *et al*., 2006; Klymus & Gerhardt, 2012) and fish (Bossu & Near, 2009; Nevado *et al*., 2011), and highlights the usefulness of introgressed mitochondrial genomes when inferring evolutionary history.

Although important information on the evolutionary history of lineages can be retained in mitochondrial genomes impacted by introgression and gene capture, it is worth noting that this utility may be influenced by the causes of introgression. If introgression is strongly driven by selection (e.g. through sex-biased hybridization or adaptive selection on mtDNA), then inferences may be different than if it is purely neutral (see Toews & Brelsford, 2012). Although the causes of mitochondrial introgression in the *H. eximia* group are unknown, they may be a result of sex-biased asymmetry (Klymus *et al*., 2010; Klymus & Gerhardt, 2012) or adaptive introgression (Bryson *et al*., 2010). As argued by Toews & Brelsford (2012), future research is needed to identify the drivers of mitochondrial introgression to further address questions about how demographics and natural selection might influence the evolutionary history of species groups prone to introgression.

CONCLUSIONS

This study adds to a growing list of species groups that demonstrate phylogenetic discord resulting from mtDNA introgression and capture (e.g. Bossu & Near, 2009; Leaché, 2010; Reid *et al*., 2012; Ruane *et al*., 2014). However, by incorporating the congruent and incongruent locus-specific phylogenetic estimates with the geographical distribution of mtDNA lineages, we gain valuable insight into phylogeography and evolutionary processes important to speciation. It is because of the very fact that introgression is usually limited in time and space to discrete geographical regions that the phylogeography is amenable to reconstruction through careful detective work on introgressed mtDNA genomes. We therefore suggest that including mtDNA in studies of species groups prone to introgression can sometimes provide a more complete picture of 'how evolutionary history played out' – complete with divergence and bouts of gene flow – rather than dismissing mtDNA altogether simply because it can return 'incorrect' phylogenies. These 'incorrect' phylogenies turn out to be more reflective of incorrect assumptions on the part of researchers (see Rubinoff & Holland, 2005; Edwards & Bensch, 2009) than they are of fatal problems with the underlying data.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Ultrametric trees for the *proopiomelanocortin A* (*POMC*), *tyrosinase* (*Tyr1*), and *β-crystallin* (*cryB*) genes coestimated with the nuclear DNA species tree in *BEAST. Nodes that received ≥ 0.95 Bayesian posterior probability support are depicted with black dots.

Table S1. Voucher data for treefrogs used in this study.

Table S2. Occurrences of potential introgression estimated by JML from the mitochondrial DNA species-tree output with P-values < 0.05.

SHARED DATA

Data deposited in the Dryad Digital Repository (Bryson *et al.*, 2014).