PHYLOGENETIC AND BEHAVIORAL DIFFERENTIATION IN THE CANYON TREEFROG, *HYLA ARENICOLOR*

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By

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The undersigned, appointed by the dean of the Graduate School,

have examined the dissertation entitled

PHYLOGENETIC AND BEHAVIORAL DIFFERENTIATION IN THE CANYON TREEFROG, *HYLA ARENICOLOR*

Presented by Katy E. Klymus

A candidate for the degree of

Doctor Of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

Professor H. Carl Gerhardt

Professor Lori Eggert

Professor Mark Flinn

Professor Johannes Schul

Professor Raymond Semlitsch

I dedicate this work

to my parents, for their love and support, and yes, now I am done with school;

and to my past mentors (Dr. Eric Pianka, Dr. C. Riley Nelson, Dr. Sue Margulis and Dr. Oliver Pergams) who showed me the beauty and fun of science, encouraged my curiosity and graciously welcomed me into research. The joy of research is not in finding all the answers but in finding more questions to ask.

“But nature is a stranger yet;
The ones that cite her most
Have never passed her haunted house,
Nor simplified her ghost.
To pity those that know her not
Is helped by the regret
That those that know her, know her less
the nearer her they get.”

--Emily Dickinson

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ABSTRACT

My research interests lie in understanding the process of speciation. In my dissertation I set out to do this by examining the first step in potential speciation, population differentiation. By looking at both phenotypic and genetic differences among populations we can hypothesize about the evolutionary forces that have led to observed phenotypic differentiation. In general, if trait divergence and genetic divergence exhibit similar variability, we may be able to infer neutral genetic drift to explain the degree of variation. If, on the one hand, populations exhibit high trait variation relative to genetic variation, we may invoke divergent selection acting on those traits. If populations have low phenotypic variation but exhibit large genetic differences, then selection may be acting to conserve a particular trait variant.

In my dissertation research I examined both genetic and behavioral differentiation within the canyon treefrog, *Hyla arenicolor*. The canyon treefrog is an excellent system for such a study because it is a widespread, abundant species ranging from the southwestern USA through southern Mexico. Recent work has shown that USA populations exhibit surprisingly high levels of mitochondrial sequence (mtDNA) divergence. The degree of divergence suggested that *H. arenicolor* might in fact be composed of a cryptic species complex. Of particular interest was the observation that divergence in male advertisement calls was not readily apparent among genetically distinct lineages in the USA. Furthermore, call differentiation was documented in the

south Mexican portion of the range (Balsas Basin); however, the phylogenetic relationships among the Mexican populations was unknown.

My goals were to document range-wide variation in advertisement calls, to understand why call and genetic variation do not coincide among USA populations, and to assess the role of female preference in promoting behavioral reproductive isolation and future divergence.

My work demonstrated that among the USA lineages advertisement calls did vary but the magnitude of the differences is unlikely to promote assortative mating by lineage through female preference of calls. By contrast, mitochondrial sequence divergence was remarkably high (13% in cytochrome-b). One might explain this observed pattern of large genetic divergence accompanied by small phenotypic variation as a result of selection acting either to conserve an ancestral call type or to converge upon a call type. However, before investigating these potential hypotheses, I re-examined the phylogeny of the canyon treefrog and it sister species group, *H. eximia,* using both mtDNA and nuclear data (exon sequence, AFLP). My results support an entirely different explanation. I found evidence for past hybridization and mtDNA introgression between two *H. arenicolor* populations and the sister species, *H. eximia*. Nuclear data suggest that some lineages of the canyon treefrog with highly divergent mtDNA actually appear undifferentiated from neighboring lineages. Advertisement call differences better reflect the inferred nuclear phylogeny. These results suggest that drift rather than selection plays a more important role in call differentiation within this system.

I also described range-wide variation in the advertisement call of the canyon treefrog. Relative to the USA lineages, we found biologically significant differences in

some call properties. My research included designing playback experiments that tested the call selectivity of female canyon treefrogs in both the lab and in the field. My results show that differences in pulse rate, call rate and call duration observed among the Mexican lineages were large enough to cause females from USA populations to discriminate against these calls, suggesting a role for behavioral reproductive isolation in the continued divergence of these lineages. Results also show that female canyon treefrogs discriminate strongly against the calls of the sister species, *Hyla wrightorum*, with which they hybridized with in the recent past. Further research concerning the role of ecological differences between these two species, the frequency of interspecific breeding interactions, and the strength of behavioral isolation may provide further insights into understanding why this group is susceptible to episodes of mitochondrial introgression.

Finally, I also developed genome-wide AFLP markers to evaluate fine-scale intraspecific variation of the canyon treefrog. My results (genetic and behavioral) support the conclusions of previous work that the behaviorally distinct Balsas Basin lineage has diverged and evolved independently of the main canyon treefrog clade for a long time and probably warrants recognition as a new species. The AFLP markers also resolve relationships within the main canyon treefrog group. Overall, my results provide insight into the complex evolutionary history of this group, have implications for the study of character evolution in this group, and emphasize the need for phylogeographic studies to expand sampling to include closely related, syntopic species.

CHAPTER 1 INTRODUCTION

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A fundamental topic of research in evolutionary biology is the formation of new species. Preceding such studies, however, one must first define what a species is, which in itself is a difficult task. Many species concepts have been formulated, each definition having its own strengths and weaknesses (Coyne & Orr, 2004). For instance species concepts based on reproductive isolation will not work for asexual taxa; instead definitions that delineate clusters based on ecological adaptive zones may be more appropriate for such taxa. Clearly a single species concept will not suffice to clearly demarcate biological diversity. Nevertheless, one concept that has played a dominant role in current speciation research is the Biological Species Concept, which is defined by reproductive isolation. Formulated during the Modern Synthesis and the work of Mayr (1942) and Dobzhansky (1937), the biological species concept defines species as "…groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such populations" (Mayr, 1942).

Thus many studies investigating speciation among sexually reproducing taxa often apply the Biological Species Concept (BSC). Because reproductive isolation defines the BSC, a key component of speciation studies is understanding what barriers prevent species or taxa from interbreeding. Both prezygotic and postzygotic isolating barriers reduce gene flow among taxa (Dobzhansky, 1937; Coyne & Orr, 2004). The striking behaviors and morphologies associated with many secondary sexual traits are often thought to play a role in reproductive isolation (Darwin, 1859). Thus systems that exhibit divergence in such traits are often examined for prezygotic behavioral reproductive isolation (Ryan et al., 1996). Besides assessing trait divergence and

reproductive isolation, another goal of these studies is to understand the evolutionary forces that lead to trait divergence and population isolation. Historically, natural selection and genetic drift were believed to be the main drivers of divergence in such traits among allopatric populations, or reinforcement via natural selection in cases of secondary contact (Mayr, 1942; Dobzhansky, 1937). More recently evidence also suggests that sexual selection can lead to rapid divergence among these traits and ultimately drive speciation (Lande, 1981; West-Eberhard, 1983; Panhuis et al., 2001).

Since Blair's (1958) pioneering work on anuran acoustic communication, anurans have become a model system for studying speciation. His work initiated extensive research in speciation and behavior (Littlejohn, 1965; Ryan & Rand, 1993; Cocroft & Ryan, 1995; Márquez, 1995; Gerhardt & Huber 2002; Lemmon & Lemmon, 2009). Male anurans produce advertisement calls that attract females for mating. Of importance in these studies is the ability to analyze variation in both signals (calls) and in receiver (female) preferences because reproductive isolation will depend on the coevolution of both components (Endler & Houde, 1995). For many anuran species studying signal and preference variation is fairly tractable. Female preference can be assessed through playback experiments and the use of synthetic or manipulated natural calls. Although most studies have focused on interspecific differences in call and preference to understand evolutionary forces acting on trait divergence, more recent work has begun to assess within-species divergence (Ryan et al, 1996; Heyer & Reid, 2003; Pröhl et al., 2006; Boul et al., 2007). Intraspecific studies that focus on divergence among populations in traits likely to promote reproductive isolation allow us to examine the first potential steps in speciation (Tregenza, 2002). Investigating intraspecific, geographic trait variation

on diverse taxa from spiders and insects to amphibians and birds has revealed how the forces of natural selection, drift and sexual selection have played roles in trait divergence, population differentiation and ultimately speciation (Gleason & Ritchie, 1998; Masta & Madison, 2002; Swallow et al., 2005; Irwin et al., 2008).

A crucial component to many of these studies is assessing the correspondence between trait and genetic divergence. Positive covariance between neutral genetic markers and trait divergence suggests that populations are diverging through random genetic drift (Ruegg et al., 2006; Hankison & Ptacek, 2008). On the other hand, if trait divergence does not correspond with genetic divergence, then selection may be acting on phenotypic variation. For instance, a high degree of molecular divergence without a similar degree of trait divergence may indicate that conservative/ convergent selection is acting on traits or that a balance between natural and sexual selection influences trait divergence (Emerson & Ward, 1998; Swallow et al., 2005; Irwin et al., 2008). If trait divergence is higher that expected based on genetic differentiation, divergent selection (either natural or sexual) plays a strong role in trait and population divergence as inferred in jumping spiders (Masta & Madison, 2002) and frogs (Boul et al., 2007). To compare genetic and trait evolution, these studies use molecular genetic tools to determine population genetic divergence and phylogenetic relationships. They then assess patterns and correlations between genetic and trait divergence.

Such studies focus on species with large ranges and/or limited dispersal as detection of population genetic structure is more likely. The large range of the canyon treefrog, *Hyla arenicolor*, makes this species an excellent system for such studies. Ranging from the southwestern USA to south central Mexico, the canyon treefrog

inhabits rocky, canyon streams in oak-pine forests (Pierce, 1968; Duellman,1970). Although canyon treefrogs have lower rates of evaporative water loss than other frogs (Preest et al., 1992), they are still believed to have limited dispersal abilities, especially across the large expanses of desert separating their mesic, montane habitat (Barber, 1999a). Thus due to their wide range and limited movement, it is expected that populations should exhibit strong genetic and phenotypic geographic variation. As in other hylids, males produce advertisement calls during the breeding season (April-May, and the monsoon season from late June through August). Geographic variation in advertisement calls was previously examined by Pierce (1968). Of particular interest was a group of populations that he termed the southern call race found in the Balsas Basin of Mexico. The southern call race was described as having large differences in call duration and call period relative to all other *H. arenicolor* populations, labeled as the northern call race (Pierce, 1968). However, no genetic studies had assessed range-wide genetic variation. Among USA populations of the canyon treefrog, large mtDNA sequence divergences are observed (Murray, 1997; Barber, 1999a; Barber, 1999b). Both Barber and Murray found strong genetic structuring among populations, and interestingly the degree of sequence divergence observed was extremely high. Barber found interspecific levels of cytochrome-b sequence divergence (9-13%) among USA lineages (1999a). Based on these findings, large differences in male advertisement call might be expected.

My dissertation begins by examining whether or not this expectation holds true among the USA lineages. I then expand my study to include both phylogenetic and behavioral sampling throughout the range. My research aimed to assess genetic and behavioral differentiation (male advertisement call and female preference) among

populations throughout the range of distribution. I wanted to then compare genetic and behavioral patterns to see whether drift or selection was the main driver of trait differentiation. Finally, I wanted to examine the potential for further divergence among populations by assessing female preference for different call types. Overall this study adds substantially to previous work on the canyon treefrog by using both molecular phylogenetics and behavioral analyses to investigate population divergence and the potential for speciation in this wide-ranging species.

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CHAPTER 2

MOLECULAR PATTERNS OF DIFFERENTIATION IN CANYON TREEFROGS (*HYLA ARENICOLOR*): EVIDENCE FOR INTROGRESSIVE HYBRIDIZATION WITH THE ARIZONA TREEFROG (*H. WRIGHTORUM*) AND CORRELATIONS WITH ADVERTISEMENT CALL DIFFERENCES

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ABSTRACT

Detection of genetic and behavioral diversity within morphologically similar species has led to the discovery of cryptic species complexes. We tested the hypothesis that USA populations of the canyon treefrog (*Hyla arenicolor*) may consist of cryptic species by examining mate-attraction signals among three divergent clades defined by mtDNA. Using a multi-locus approach, we re-analyzed phylogenetic relationships among the three clades and a closely related, but morphologically and behaviorally dissimilar species, the Arizona treefrog (*H. wrightorum)*. We found evidence for introgression of *H. wrightorum'*s mitochondrial genome into *H. arenicolor*. Additionally, the two-clade topology based on nuclear data is more congruent with patterns of call variation than the three-clade topology from the mitochondrial dataset. The magnitude of the call divergence is probably insufficient to promote isolation of the nuclear-DNA defined clades should they become sympatric, but further divergence in call properties significant in species identification could promote speciation in the future.

INTRODUCTION

Theories of speciation lead to the general expectation that genetic differentiation among species or populations of wide-ranging species will be reflected in phenotypic traits. In particular, evolutionary biologists focus on differences in sexually selected traits because of their role in promoting reproductive isolation and speciation (Ryan et al., 1996; Tregenza & Butlin, 1999; Masta & Madison, 2002; Swallow et al, 2005). Indeed, differences in reproductive behaviors in animals breeding at the same time and place

have often led to the discovery of cryptic species, defined here as morphologically similar species with high levels of genetic divergence (Stein, 1963; Zink & Johnson, 1984; Shaw, 2000; Bickford et al., 2007; Lemmon et al., 2007).

However, this correspondence of genetic and behavioral divergence between species does not always apply, and in fact comparisons of genetic and phenotypic divergence among populations of a single wide-ranging species often show a diversity of patterns (Coyne & Orr, 2004; Tregenza, 2002). Although some studies find the expected correlation (Christianson et al., 2005; MacDougall-Shackleton & MacDougall-Shackleton, 2001), many others do not (Tregenza & Butlin, 1999). For example, geographic distance was a better predictor of behavioral isolation than genetic distance in the plethodontid salamander, *Desmognathus ochrophaeus* (Tilley et al., 1990) and in the túngara frog, *Physalaemus pustulosus* (Ryan et al., 1996; Pröhl et al., 2006). Sexual selection was found to drive trait divergence in both jumping spiders (Masta & Maddison, 2002) and frogs (Boul et al., 2007). Alternatively, sexually selected traits sometimes show less diversification than expected among genetically distinct populations, as in fanged frogs (Emerson & Ward, 1998) and stalk-eyed flies (Swallow et al., 2005). Thus within a species, complex interactions between evolutionary forces acting on local populations, such as counterbalancing natural and sexual selection, often leads to a discordance between genetic and phenotypic divergence.

Our original goal was to discover if behavioral divergence (advertisement calls) among populations of the canyon treefrog, *H. arenicolor,* corresponds to previously reported genetic divergence. Barber (1999) found three geographically isolated, genetically distinct, mitochondrial lineages within the USA portion of this species'

distribution: mean sequence divergences (ranging between 9.2 and 13.3%) were of sufficient magnitude to suggest the presence of three, allopatrically distributed species. Differences in advertisement calls have previously identified cryptic species of gray treefrogs (*H. versicolor* and *H. chrysoscelis*: Bogart & Wasserman, 1972; Ptacek et al., 1994; Holloway et al., 2006) and corroborated genetic divergence in chorus frogs (genus *Pseudacris*: Lemmon et al., 2007). Thus we sought to determine if call properties known to promote reproductive isolation in frogs differed among the three mtDNA-defined clades of *H. arenicolor* (Barber, 1999). If so, were the differences likely to be sufficient to promote between-clade discrimination or did they merely reflect relatively minor consequences of drift and selection expected in geographically isolated populations?

During the course of sampling calls, several studies were published that reinforced the need to corroborate phylogenetic patterns derived from mtDNA (Shaw, 2002; McGuire et al., 2007; Good et al., 2008). Molecular studies based on a single marker can be confounded by the effects of ancestral polymorphism, lateral gene transfer (introgression), or different rates of evolution among markers. Studies including multiple loci can help to avoid misinterpretation of phylogenetic relationships resulting from these effects (Moore, 1995; Ballard & Whitlock, 2004). We therefore compared mtDNA and nuclear markers.

We will show that the three-clade structure proposed by Barber (1999) is confounded by introgressive hybridization with another treefrog species, *H. wrightorum*. Moreover, patterns of call differentiation among *H. arenicolor* better fit a two-clade structure indicated by the nuclear data. Although the magnitude of the behavioral differences between the two nuclear-defined clades would almost certainly be insufficient

to promote between-clade reproductive isolation at this time, our results identify the groups of populations with the greatest potential for speciation. Our results serve to emphasize the necessity of using multiple genetic markers when characterizing patterns of genetic differentiation.

METHODS

Recording and Tissue Sampling

During 2000-2007 we recorded the advertisement calls of 74 males of *H. arenicolor* in 11 populations from throughout the USA range (Table 2-1; Fig. 2-1). For the genetic analyses, tissue from 28 individuals of *H. arenicolor* (see Fig. 2-1 for localities), *H. wrightorum*, *H. eximia*, and three outgroup species, *H. femoralis, H. avivoca*, and *H. chrysoscelis* were analyzed (Table 2-2). We analyzed the calls of three of the sixteen *H. arenicolor* used in the genetic analysis. Call data were collected from all of the same populations as the remaining individuals sampled for the genetic analysis.

Call Analyses

For each male, at least ten consecutive advertisement calls were recorded between 2100 and 0100 hrs using either a Sony stereo cassette recorder (TC-D5M), a Tascam DAT recorder (DA-P1), or a solid-state Marantz digital recorder (PMD670). A Sennheiser directional microphone (ME-66) with windscreen was positioned at 50-100 cm from the calling frog. The frequency-response of all three recorders was flat within ± 3 dB over the range from 30-17000 Hz, and the speed variation (which limits the accuracy of the measurement of temporal properties) was less than 0.06%. After each recording, we measured body temperature to the nearest 0.1° C using a Weber mercury

quick-read cloacal thermometer. Calls recorded on tape cassettes were first digitized at a sampling rate of 44 kHz using Sound Edit 16 v.2 (Macromedia, 1996). We analyzed five sequential advertisement calls from each recording session using the Raven 1.2.1 software package (Cornell Lab of Ornithology, 2003); temporal properties were measured from oscillogram displays and spectral properties, from power spectra (Hamming window, FFT length = 1024 samples).

The advertisement call of *H. arenicolor* is composed of a series of pulses (Fig. 2-2a). The acoustic energy is concentrated in two broad spectral peaks; a series of sidebands are spaced at frequency intervals equal to the periodicity of the repeating waveform (Fig. 2-2c). Each pulse also possesses a sub-pulse structure (Fig. 2-2b). In addition to the high-frequency peak, which usually contains the greatest amplitude, we analyzed five temporal call variables: number of pulses; pulse duration; call period; pulse rate; and pulse rise-time (Fig. 2-2). We chose to examine these six call variables because they are known to be important for species recognition in other frogs (Gerhardt & Huber, 2002).

We analyzed patterns of call variation using STATISTICA 5.5 software (StatSoft 2000). Before applying parametric tests, we tested all trait distributions for normality with a Kolmogorov-Smirnov goodness-of-fit test. Where necessary, data were log-transformed to meet assumptions of homoscedasticity and normality. Several call characteristics were found to be temperature-dependent, and so values for these characteristics were temperature corrected to 18° C using the results of a linear regression of the call variable on temperature.

We used a forward-stepwise discriminant function analysis to determine whether advertisement call variables were effective in predicting membership in the three mtDNA clades proposed by Barber (1999), because this procedure allows for the a priori consideration of groups (James & McCulloch, 1990). The canonical variates that are generated summarize multivariate trait variation and produce corrected distances among groups. However, because call characteristics are frequently correlated, we also conducted a principal components analysis to generate a smaller number of uncorrelated variables, which were then subsequently used in a multivariate analysis of variance. Examination of the factor loadings allowed us to determine which particular call variables contributed most to behavioral divergence between the mitochondrial clades.

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted from EtOH-preserved toe and liver tissue with the DNeasy Tissue Kit (Quiagen, Inc., Valencia, CA). An approximately 2.5 kb segment of mitochondrial 12S and 16S genes and the intervening valine tRNA were amplified with a set of overlapping primers (Table 2-3, Goebel et al., 1999; Appendix A). Three nuclear gene fragments (a single exon of Rag 1, a segment from exon 4 of Rhodopsin, and a segment of an anonymous marker G11T) were amplified via polymerase chain reaction (PCR) and selected primer pairs (Table 2-3).

PCR amplifications of the mitochondrial fragments were performed with the following thermocycler protocol: 2 min at 94° C, denaturing for 30 sec at 94° C, annealing for 30 sec at 47° C, extension for 1 min at 72° C, and final extension for 7 min at 72° C; denaturing, annealing and the first extension stage were cycled 35 times. Nuclear fragments were amplified under the following conditions: 10 min at 95° C,

denaturing for 1 min at 95° C, annealing at 55° to 58° C (see Table 2-3) for 1 min, extension for 1 min at 72° C, and a final extension at 72° C for 7 min; denaturing, annealing and the first extension stage were cycled 35-40 times. Products were visualized with a 2% agarose gel, and products were purified using a QIAquick PCR purification kit (Quiagen, Inc., Valencia, CA). Sequencing reactions were completed with an automated DNA sequencer (ABI 3730 and 3700 analyzers).

Alignment and Phylogenetic Analysis

Mitochondrial fragments were combined to give a contig of 2,453 bp. Alignment was performed using Clustal X; within the ingroup, very few regions were difficult to align, and these were adjusted manually to minimize the inferred number of evolutionary changes. Nuclear sequences were aligned and edited using SEQUENCHER 4.5 and rechecked by eye. Heteozygous sites were left as ambiguous and only one sequence per individual was produced per nuclear loci. One *H. arenicolor* individual, HAR52, was found to be heterozygous between mtDNA Clades 1 and 2. No heterozygous individuals between *H. arenicolor* and *H. wrightorum* were observed. The three nuclear fragments were run in separate maximum parsimony and maximum likelihood analyses. Nuclear fragments were then concatenated to give 960 bp.

Phylogenetic analyses were conducted by creating trees using maximum parsimony (MP) and maximum likelihood (ML) methods. Maximum parsimony trees were generated using PAUP 4.0b10 (Swofford, 2002). Both the mtDNA and nuclear data sets were analyzed using heuristic searches with 100 random addition sequences and TBR branch swapping. Nodal support values were obtained through bootstrapping with 2000 pseudoreplicates. For the maximum likelihood analysis, Modeltest 3.7 (Posada &

Crandall, 1998) was used for each dataset to select a best-fit model of nucleotide substitution. For the mtDNA, the selected model according to the Akaike Information Criterion (AIC) was: GTR+G+I. The HKY+I model was selected for the concatenated nuclear dataset. The program Phyml (Guindon & Gascuel, 2003) was used for the maximum likelihood analysis with 2000 bootstrap pseudoreplicates.

RESULTS

Call Analysis

The average values of the call properties produced by males in each of the three mtDNA-defined clades are summarized in Table 2-4. Several of the call properties are quite similar among clades, with average differences less than 10% (pulse duration, pulse repetition rate, pulse rise time). Three call characteristics are more variable between mitochondrial clades: number of pulses, call period, and high-frequency peak. Nevertheless, there was extensive overlap in call properties between these groups in all pairwise comparisons.

Discriminant function analysis using the six call variables discriminated among the three mitochondrial clades. All six variables were retained in the significant model (Wilks' $\Lambda = 0.308$; $F_{12,132} = 8.81$; $P < 0.0001$). Malahanobis distances confirm that calls produced by males from mtDNA Clade 2 are more distant from calls in Clades 1 and 3 than they are from each other in terms of multivariate call space (Table 2-5).

Many of the call characteristics analyzed are significantly intercorrelated; therefore, the six call variables were subjected to a principal component analysis. The principal axis method was used to extract the components, and this procedure was

followed by a varimax (orthogonal) rotation. The first two components displayed eigenvalues greater than 1, together accounting for 54.7% of the total variance. Corresponding factor loadings are presented in Table 2-6. In interpreting the rotated factor pattern, an item was considered to load on a given component if the factor loading was 0.60 or greater. Using this criterion, number of pulses, call period and high-frequency peak were found to load on the first component. The second component is comprised of pulse repetition rate and pulse duration.

Examination of the factor scores showed clearly that there was extensive overlap in call structure between mtDNA Clades 1 and 3 and that there was less overlap in the calls of these two clades with mitochondrial Clade 2 (Fig. 2-3). Using the two principal components as variables in a multivariate analysis of variance, we confirmed that males in the three mtDNA clades produced advertisement calls with significantly different call characteristics (Wilks' $\Lambda = 0.52$, Rao's $R_{4,140} = 13.51$, $P < 0.0001$). Subsequent univariate analyses showed that the first principal component differed significantly among mtDNA-defined clades ($F_{2,71} = 24.6$, $P < 0.0001$, Table 2-7). Post-hoc analyses indicate that males in Clade 2 produced advertisement calls that were significantly different from calls produced by males in Clades 1 and 3 (Tukey Honest Significant Difference Test for Unequal N, P's < 0.001). Males in mtDNA Clade 2 produced short calls of high frequency at a rapid rate compared to males in the other clades. The second principal component did not differ significantly among mitochondrial clades ($F_{2,71} = 2.83$, $P = 0.066$).

Phylogenetic Analyses

mtDNA analysis

Both analyses from the mtDNA sequence dataset confirm the phylogenetic structure described by Barber (1999), in which individuals fall into one of three distinctive genetic clades. Furthermore, as in Barber's work, all *H. arenicolor* Clade 3 individuals group with individuals of *H. wrightorum*. Clade 3 individuals are confined to the Grand Canyon, with Clade 1 individuals found both north and south of it (Fig. 2-1). The left side of Figure 2-4 shows the ML tree for the mitochondrial data set.

Nulcear DNA analysis

Tests for incongruency among the three nuclear fragments were run using the incongruence length difference (ILD; Farris et al., 1994) and Shimodaira-Hasegawa (SH; Shimodaira & Hasegawa, 1999) tests. The ILD test found all three fragments to be congruent (P's > 0.05). The SH test found the Rhod and Rag fragments to be congruent with one another (P = 0.213) ; however, both were significantly incongruent with the third fragment, G11T (P's < 0.05). Despite this inconsistency, all three nuclear fragments strongly support the removal of mtDNA Clade 3 individuals from the mitochondrial grouping with *H. wrightorum* and place them among the *H. arenicolor* mtDNA Clade 1 individuals. Some of the discrepancy is probably due to the placement of HAR52, the individual that is heterozygous at one locus (Rag) between *H. arenicolor* Clades 1 and 2. This individual's placement has a low bootstrap value of 58. Furthermore the *H. arenicolor* individual from west Texas, HAR 3468, had a few unique substitutions, making its placement among the other *H. arenicolor* individuals less certain (bootstrap value 62). Finally, the largest discrepancy was caused by the placement of *H. eximia*

individuals, in which they were either placed with *H. wrightorum* in a polytomy with the outgroup species (Rag), placed sister to *H. wrightorum* (Rhod), or placed sister to a group that included all *H. wrightorum* and *H. arenicolor* (G11T).

We assert that these incongruencies reflect the paucity of informative sites. Because all *H. arenicolor* group out from *H. wrightorum* in all three genes, we decided to concatenate the data, following Wiens (1998). He posits that combining datasets, even if incongruent, can increase the accuracy in regions that are already well supported by increasing the number of informative sites. This method may be especially helpful when the number of informative sites is low, as is in our case. Out of 960 base pairs of nuclear sequence data, only 39 base pairs were informative.

Using a concatenated data set of all three nuclear regions, both ML and MP analyses strongly support the monophyly of *H. arenicolor*. Because MP and ML analyses are similar, we only show the ML tree along with ML and MP support values (Fig. 2-4). The nuclear analyses reveal that the mtDNA Clade 3 does not group with *H. wrightorum*, but instead individuals are placed with *H. arenicolor* Clade 1 individuals. A second group within *H. arenicolor* is composed of most of the mtDNA Clade 2 individuals. Also differing from the mtDNA topology, the individual HAR51 no longer groups in with Clade 1, but is instead found in the group with mtDNA Clade 2 individuals, and this placement is strongly supported (bootstrap of 84).

DISCUSSION

This study was conducted to test the idea that genetic divergence might be paralleled by biologically significant differences in pre-mating isolating mechanisms in

three mtDNA-defined clades, consisting of geographically isolated groups of populations. Our analysis of nuclear DNA, however, led to the discovery of a significant discrepancy between the nuclear and mtDNA gene trees. We hypothesize that the observed incongruity was caused by mitochondrial introgression between *H. arenicolor* from mtDNA Clade 3 and *H. wrightorum.*

Considering the extreme phenotypic differences between *H. arenicolor* and *H. wrightorum*, the history and ecology of introgression between them is arguably of more evolutionary interest than the potential reproductive isolation of different *H.arenicolor* groups. *H. wrightorum* and its sister species, *H. eximia*, are the closest extant relatives of *H. arenicolor* (Hedges, 1968; Barber, 1999); they are found throughout Arizona and Mexico, with distributions mostly occurring within the range of *H. arenicolor* (Fig. 2-1). These two species differ greatly from *H. arenicolor* morphologically and behaviorally (Duellman, 1970; Gergus et al., 2004). They are smaller and have a smooth, typically green or dark brown skin compared to the bumpier gray and tan skin of *H. arenicolor.* Comparison of the waveforms reveals that the advertisement calls produced by these two species are strikingly different in temporal properties, call shape and pulse shape (Fig. 2-2). Although our study did not address variation in the advertisement calls of *H. wrightorum*, Gergus et al. (2004) found that this species' average pulse repetition rate (108 pulses/s versus 15 pulses/s) and call duration (194 ms versus 975 ms) were quite different from those of *H. arenicolor*. Mixed-species breeding choruses of *H. arenicolor* and *H. wrightorum* commonly occur in northern and central Arizona, and field observations indicate that the dominant species at the chorus changes with the time of the breeding season and local patterns of rainfall (Gerhardt, pers. obs.). Mating mistakes

involving interception of females by more abundant heterospecific males are well documented in anuran amphibians (Lamb & Avise, 1986; Gerhardt & Huber, 2002). Thus the possibility of mismatings between these two species is likely. Even though *H. wrightorum* is currently not found within the Grand Canyon, where the introgressed clade is found, dispersal of introgressed individuals from adjacent sympatric areas or the extinction of *H. wrightorum* from the Grand Canyon are likely scenarios.

Discrepancies between mtDNA and nuclear gene trees are often credited to either ancestral polymorphisms and incomplete lineage sorting, or introgressive hybridization (Moore, 1995; Avise, 2000). The first cause seems unlikely here, because of the long branch lengths separating the various groups. In both the mitochondrial and nuclear analyses, branch lengths subtending each major lineage are longer than branch lengths within groups. Thus, the mtDNA haplotypes of *H. wrightorum* and Clade 3 *H. arenicolor* are less divergent from one another than from the other *H. arenicolor* mtDNA clades. In contrast, nuclear sequences of individuals from the mtDNA Clades 1 and 3 are more similar to one another than to sequences of *H. wrightorum*. We interpret this pattern as resulting from past hybridization between *H. wrightorum* and *H. arenicolor,* which led to mitochondrial introgression. Our sampling suggests that the introgression is unidirectional with the mitochondrial genome of *H. wrightorum* being introgressed into populations of *H. arenicolor* and not vice versa, as only one *H. arenicolor* clade is nested within the mitochondrial group of *H. wrightorum* and *H. eximia*.

Evidence of cytoplasmic genome (chloroplast and mitochondrial) introgression in plant and animal taxa is well documented (Dowling & Secor, 1997; Wirtz, 1999; Shaw, 2002; Arnold, 2006; Baack & Rieseberg, 2007; McGuire et al., 2007; Good et al., 2008),

and it has been found in anurans as well (Splosky & Uzzell, 1984; Lamb & Avise, 1986; Lemmon et al., 2007; Plötner et al., 2008). We did not find evidence of nuclear introgression between *H. arenicolor* and *H. wrightorum*, as no individuals shared alleles between these species. Several studies have shown mitochondrial introgression with little or no apparent nuclear introgression (Bernatchez et al., 1995; Glémet et al.,1998; McGuire et al., 2007). Indeed, Bernatchez et al. (1995) found the mtDNA of an allopatric population of brook char to be identical to that of the arctic char, even though these brook char are indistinguishable from other brook char populations in terms of morphology and nuclear DNA, a situation similar to that of the *H. arenicolor* mitochondrial Clade 3. Furthermore, the calls of *H. arenicolor* from the introgressed Clade 3 are similar to those of *H. arenicolor* from other clades, and there is no resemblance to calls of *H.wrightorum* (Fig. 2-2), indicating that the introgressed genes have not affected call structure of these populations. Mitochondrial introgression may also have occurred between clades, as one individual from the Chirachuahua mountains of southern Arizona (HAR 51) no longer grouped with mtDNA Clade 1 individuals but was well supported in its placement with mtDNA Clade 2 individuals (Fig. 2-4). The other *H. arenicolor* individuals that differed in the nuclear topology (HAR 3468 and HAR 52) had low bootstrap support and so their placement cannot be considered as evidence of introgression. More extensive sampling of *H. arenicolor* (particularly in areas where clades may potentially overlap), *H. wrightorum,* and *H.eximia*, and the use of more highly informative markers is needed to better explore and understand this introgressive event.

Our call analysis found that call differentiation better reflects the nuclear gene topology than that of the mitochondrial gene tree. Calls of males from the southern

portion of the USA range, mostly mtDNA Clade 2, differ from those of the other two mtDNA-defined clades (1 and 3), which are quite similar. Nevertheless, the magnitude of differences in call properties of known relevance to female anurans is unlikely to be sufficient to promote reproductive isolation should populations of different clades become sympatric in the near future. With regard to call properties useful for species identification in other hylid frogs, for example, consider pulse rate and dominant frequency. The average between-clade difference in mean pulse rate – the property most often used for species recognition in anurans – is of the order of 5%. Experiments using synthetic calls indicate that female hylids require a minimum difference of the order of 20% for effective discrimination (e.g., Gerhardt, 2005; review: Gerhardt & Huber, 2002). Variation in dominant frequency is correlated with body size in most hylids and many other kinds of frogs (review: Gerhardt & Huber, 2002); thus, species differences and geographic variation in this property could be the result of selection on body size as well as an effect of female choice. The mean high-frequency peak in Clade 2 is about 19% higher than that in Clades 1 and 3, but preferences based on frequency in frogs are easily abolished or reversed by differences in intensity that arise because of spatial distributions of males and females in choruses (review: Gerhardt & Huber, 2002).

There is also extensive overlap between clades in values of other call properties (Table 2-4). Thus even if discrimination of differences in any of these properties were far superior in *H. arenicolor* to that in other frog species, females would usually encounter pairs of individual males of different clades with values that would not be sufficiently different (if at all) for mate identification. Future studies examining female choice between exemplars of pre-recorded calls from the two nuclear-defined groupings should

test this conclusion directly. The use of natural vocalizations would also test for the possibility that discrimination could be based on combinations of call properties (e.g., Gerhardt & Brooks, 2009).

Our study adds to the literature on mitochondrial introgression in vertebrates, and supports the view that multiple lines of evidence are needed when inferring phylogenetic histories. We demonstrate inter-specific mitochondrial introgression between the canyon treefrog, *H. arenicolor* and its sister species *H. wrightorum*. Further sampling at the population level from these two species are required to explore the possibility of discovering more introgressive events, explore possible areas of current hybridization, and address hypotheses concerning introgression.

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Table 2-1. Populations from which male calls were recorded and number of males (N).

Population	Clade	Latitude	Longitude	N
Davis Mountains, TX	1	30.700	-103.500	2
Reynolds Creek, AZ	1	33.874	-110.992	6
Lower Workman Creek, AZ	1	33.846	-110.972	10
Parker Canyon, AZ	1	33.798	-110.967	3
Pine Creek, UT	1	37.217	-112.974	7
Madera Canyon, AZ	2	31.735	-110.883	11
Box Canyon, AZ	2	31.798	-110.777	3
Jacobson's Creek, AZ	2	32.667	-109.803	2
Ruby Road, AZ	2	31.397	-111.139	2
Diamond Creek, AZ	3	35.766	-113.373	26
Peach Springs, AZ	3	35.586	-113.435	2

Table 2-2. Locality information for specimens used in molecular analysis and GenBank numbers for sequences.

					GenBank			
					mtDNA	nDNA		
Species	Specimen	Population	Latitude	Longitude	12S, 16S	Rag	Rhod	11T
Hyla arenicolor	HAR1	Peach Springs, AZ	35.586	-113.435	GU989087	GU989059	GU944717	GU968612
Hyla arenicolor	HAR8	Diamond Creek, AZ	35.766	-113.373	GU989077	GU989049	GU944707	GU968602
Hyla arenicolor	HAR11	Lower Workman Creek, AZ	33.846	-110.972	GU989068	GU989040	GU944698	GU968593
Hyla arenicolor	HAR49	Houston Mesa, AZ	34.364	-111.282	GU989065	GU989037	GU944695	GU968590
Hyla arenicolor	HAR50	Pine Creek, UT	37.217	-112.974	GU989084	GU989056	GU944714	GU968609
Hyla arenicolor	HAR51	Rucker Canyon Road, AZ	31.753	-109.418	GU989075	GU989047	GU944705	GU968600
Hyla arenicolor	HAR52	Jacobson's Creek, AZ	32.667	-109.803	GU989067	GU989039	GU944697	GU968592
Hyla arenicolor	HAR53	Ruby Road, AZ	31.397	-111.139	GU989086	GU989058	GU944716	GU968611
Hyla arenicolor	HAR63	Colorado river mile 246, AZ	35.824	-113.648	GU989091	GU989063	GU944721	GU968616
Hyla arenicolor	HAR65	Colorado river mile 273, AZ	36.096	-113.921	GU989092	GU989064	GU944722	GU968617
Hyla arenicolor	HAR3468	Davis Mountains, TX	30.685	-104.078	GU989080	GU989052	GU944710	GU968605
Hyla arenicolor	HARB43	Santa Rita Field Station, AZ	31.739	-110.866	GU989066	GU989038	GU944696	GU968591
Hyla arenicolor	HARA20	Box Canyon, AZ	31.798	-110.777	GU989072	GU989044	GU944702	GU968597
Hyla arenicolor	HARB28	Cave Canyon, AZ	31.709	-110.771	GU989070	GU989042	GU944700	GU968595
Hyla arenicolor	HARB9a	Peach Springs, AZ	35.586	-113.435	GU989076	GU989048	GU944706	GU968601
Hyla arenicolor	HARB33	Diamond Creek, AZ	35.766	-113.373	GU989071	GU989043	GU944701	GU968596
Hyla eximia	HEX31	Jalisco, MX	19.978	-103.261	GU989088	GU989060	GU944718	GU968613
Hyla eximia	HEX32	Jalisco, MX	19.978	-103.261	GU989074	GU989046	GU944704	GU968599
Hyla eximia	HEX370	Jalisco, MX	20.917	-103.033	GU989083	GU989055	GU944713	GU968608
Hyla eximia	HEX369	Jalisco, MX	20.917	-103.033	GU989078	GU989050	GU944708	GU968603
Hyla wrightorum	HWR58	Cochise Co., AZ	31.494	-110.403	GU989085	GU989057	GU944715	GU968610
Hyla wrightorum	HWR59	Coconino Co., AZ	34.286	-110.858	GU989069	GU989041	GU944699	GU968594
Hyla wrightorum	HWR60	Gila Co., AZ	34.253	-110.844	GU989089	GU989061	GU944719	GU968614
Hyla wrightorum	HWR33	Pinetop, AZ	34.13	-109.932	GU989079	GU989051	GU944709	GU968604
Hyla wrightorum	HWR34	Pinetop, AZ	34.13	-109.932	GU989081	GU989053	GU944711	GU968606
Hyla avivoca	HAV	Macon Co., AL	32.476	-85.607	GU989090	GU989062	GU944720	GU968615
Hyla chrysoscelis	HCH61	Phelps Co., MO	37.616	-91.984	GU989082	GU989054	GU944712	GU968607
Hyla femoralis	HFE3858	Chatham Co., GA	31.999	-81.120	GU989073	GU989045	GU944703	GU968598

Table 2-3. Primers used for amplification and/ or sequencing.

Primer Name	Primer Sequence (5'-3')	Annealing Temperature (° C)	Source
MtDNA			
MVZ-59	ATAGCACTGAAAAYGCTDAGATG	47	(Goebel et al., 1999)
tRNAVal	GGTGTAAGCGARAGCTTTKGTTAAG	47	(Goebel et al., 1999)
12L1	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	47	(Goebel et al., 1999)
16sh	GCTAGACCATKATGCAAAAGGTA	47	(Goebel et al., 1999)
12sm	GGCAAGTCGTAACATGGTAAG	47	(Pauly et al., 2004)
16sa	ATGTTTTTGGTAAACAGGCG	47	(Goebel et al., 1999)
16sc	GTRGGCCTAAAAGCAGCCAC	47	(Pauly et al., 2004)
16sd	CTCCGGTCTGAACTCAGATCACTGAG	47	(Pauly et al., 2004)
nDNA			
Rag1-C	GGAGATGTTAGTGAGAARCAYGG	55	(Biju & Bossuyt, 2003)
Rag1-D	GCTGCATTTCCRATRTCACAGTG	55	(Biju & Bossuyt, 2003)
Rhod1A	ACCATGAACGGAACAGAAGGYCC	57	(Bossuyt & Milinkovitch, 2000)
Rhod1C	CCAAGGGTAGCGAAGAARCCTTC	57	(Bossuyt & Milinkovitch, 2000)
11T_84F	TGGAGTACCCCTTTAAATCTGAAT	58	(Holloway et al., 2006)
11T_388R	ATAAAGTGCATAAGTAAGTAAAAGTGAA	58	(Holloway et al., 2006)
11T-A-F	ACCCTAAAAGAGCAAACGTC	55	(this study)
11T-A-R	GGCCCCTGGTCAGAGATAC	55	(this study)

Table 2-4. Mean, standard deviation and average percentage differences for each call variable between three mitochondrial clades.

Character	Clade 1 (28)	Clade 2 (18)	Clade 3 (28)	Avg% Difference
Number of pulses	17.9 ± 2.6	15.0 ± 2.5	15.8 ± 1.8	11.0
Pulse duration (ms)	39.4 ± 4.9	42.3 ± 3.5	37.0 ± 3.4	8.5
Call period (sec)	3.600 ± 0.658	2.816 ± 0.450	3.662 ± 0.736	15.5
Pulse rate (1/sec)	16.30 ± 1.06	15.15 ± 1.12	15.53 ± 0.90	4.7
Pulse rise time (%)	69.1 ± 4.9	69.6 ± 5.2	65.4 ± 3.8	4.0
High-frequency peak (Hz)	2093.2 ± 251.8	2487.5 ± 206.9	2154.8 ± 294.1	10.7

Table 2-5. Results from discriminant function analysis using six call characteristics. Squared Mahalanobis distances are presented below the diagonal. F- and P-values are presented above the diagonal.

	Clade 1	**Clade 2**	**Clade 3**
Clade 1	-	10.3(<0.00001)	6.3 (0.00003)
Clade 2	6.34	-	11.0 (<0.00001)
Clade 3	3.00	6.78	-

Table 2-6. Factor loading for two principal components derived from principal components analysis using varimax rotation. Loadings of greater than 0.60 are indicated in bold.

Character	***PC1***	***PC2***
Number of pulses	**0.62**	0.31
Call period	**0.76**	-0.24
Pulse repetition rate	0.19	**0.80**
High-frequency peak	**-0.69**	-0.30
Rise time	-0.39	0.32
Pulse duration	0.05	**-0.80**
Eigenvalue	1.85	1.43
% Total Variance	30.9	23.8

Table 2-7. Mean values for principal components in each of the three clades. Superscript letters indicate significant differences between mitochondrial clades ($P<0.001$).

	Clade 1	Clade 2	Clade 3
PC1	0.50 [a]	-1.10 [b]	0.21 [a]
PC2	0.30 [a]	-0.40 [a]	-0.04 [a]

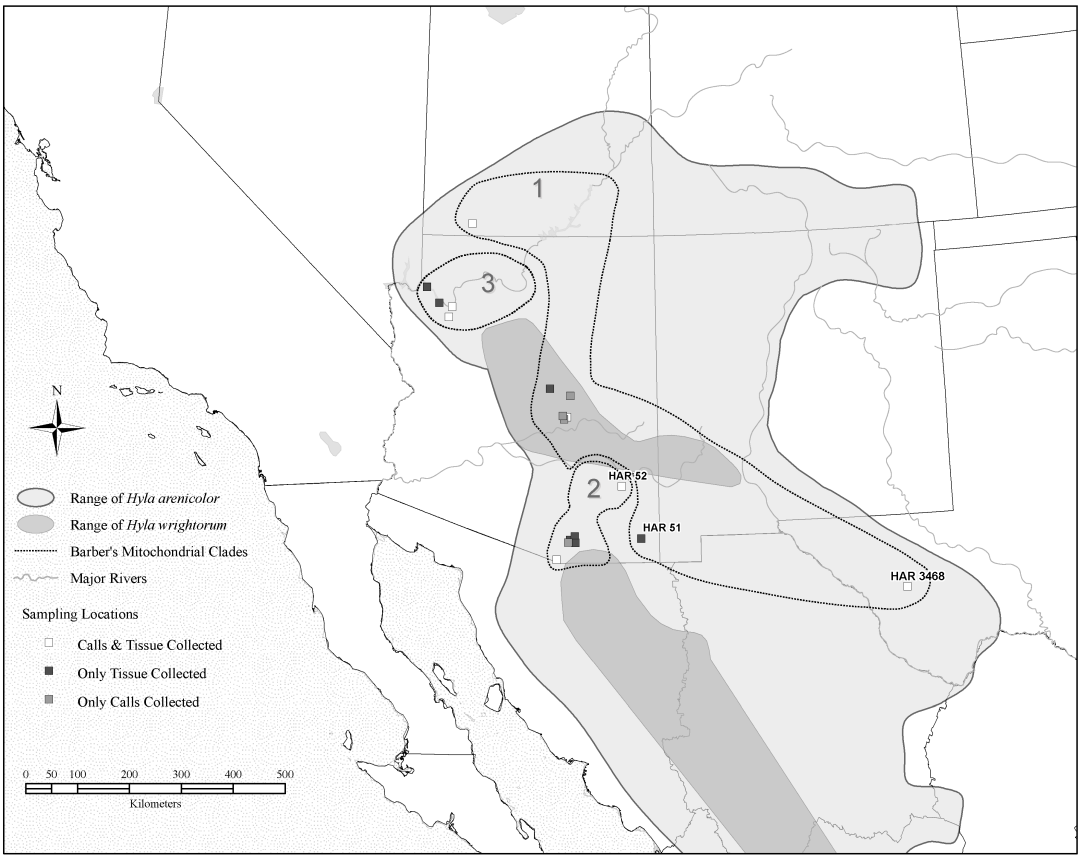

Figure 2-1. Map showing the USA portion of the *H.arenicolor* species' range. Dashed lines indicate the distribution of Barber's three mitochondrial clades, whose identities correspond to the number. The boxes indicate locations where tissue and male advertisement calls were sampled. HAR 51, HAR 52, and HAR 3468 correspond to the locality where these tissue samples were collected from individuals other than those in Clade 3 that changed position in the nuclear gene tree relative to the mtDNA topology.

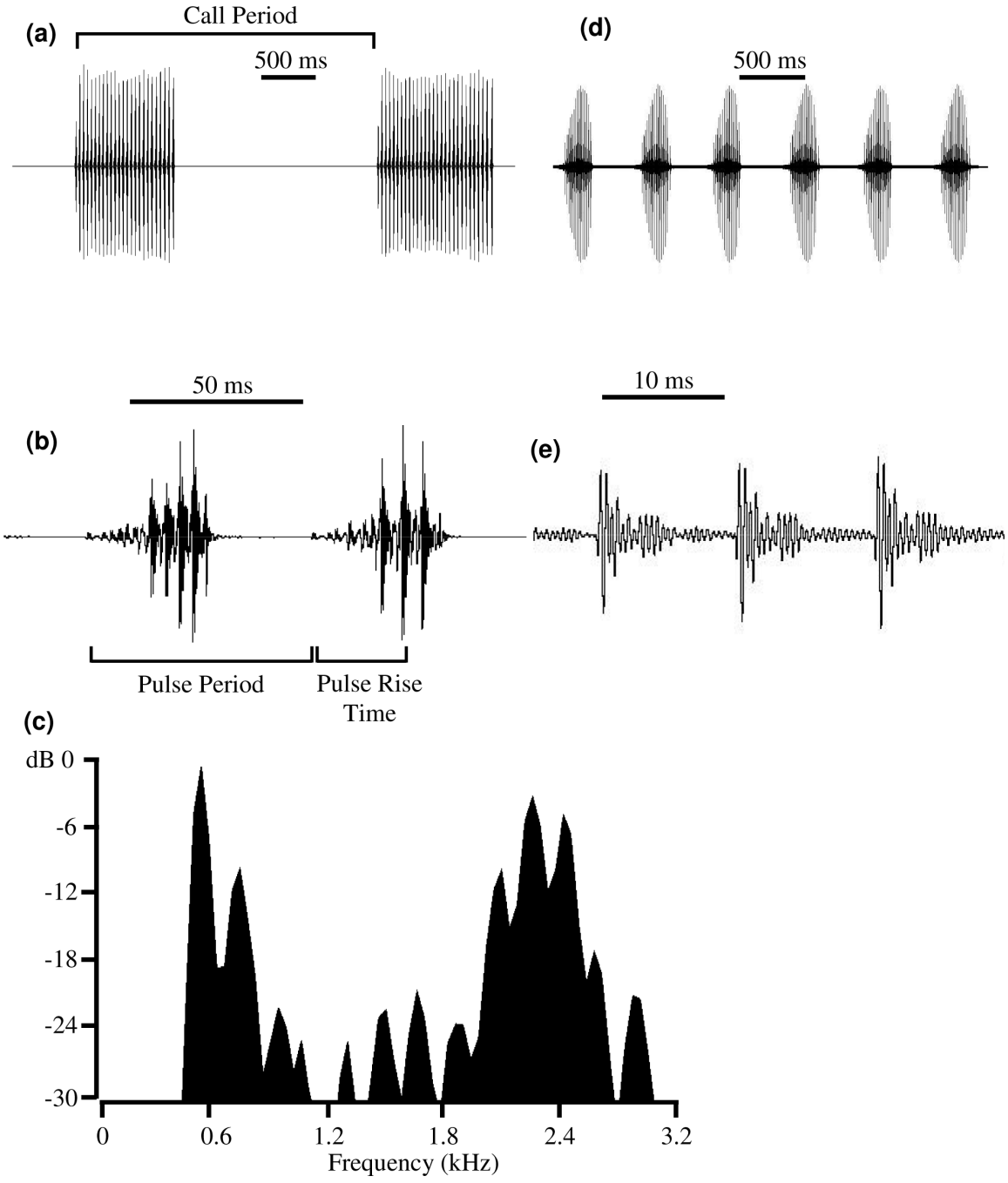
(a)
Call Period
500 ms
(d)
500 ms
50 ms
(b)
10 ms
(e)
Pulse Period
Pulse Rise Time
(c)
dB 0
-6
-12
-18
-24
-30
0
0.6
1.2
1.8
2.4
3.2
Frequency (kHz)

Figure 2-2. Diagram of the call traits used in the analysis.
(a) Oscillogram of two *H. arenicolor* advertisement calls. Call period is defined as the time interval between the beginning of one call and the beginning of the subsequent call. (b) Expanded view of two pulses from within a single call showing the distinctive sub-pulse structure. The pulse rate of the call is calculated as the inverse of the pulse period, the time interval between the beginning of one pulse and the beginning of the subsequent pulse. Pulse rise time is the time interval from the beginning of a pulse to the maximum amplitude of the pulse. (c) Power spectrum of advertisement call. (d) Oscillogram of a train of *H. wrightorum* advertisement calls. (e) Expanded view of three pulses from within a single *H. wrightorum* call. Time scales are indicated within each oscillogram. A video showing male *H. arenicolor* and *H. wrightorum* calling can be seen at: http://www.biosci.missouri.edu/gerhardt/video/aren_wright.m4v.

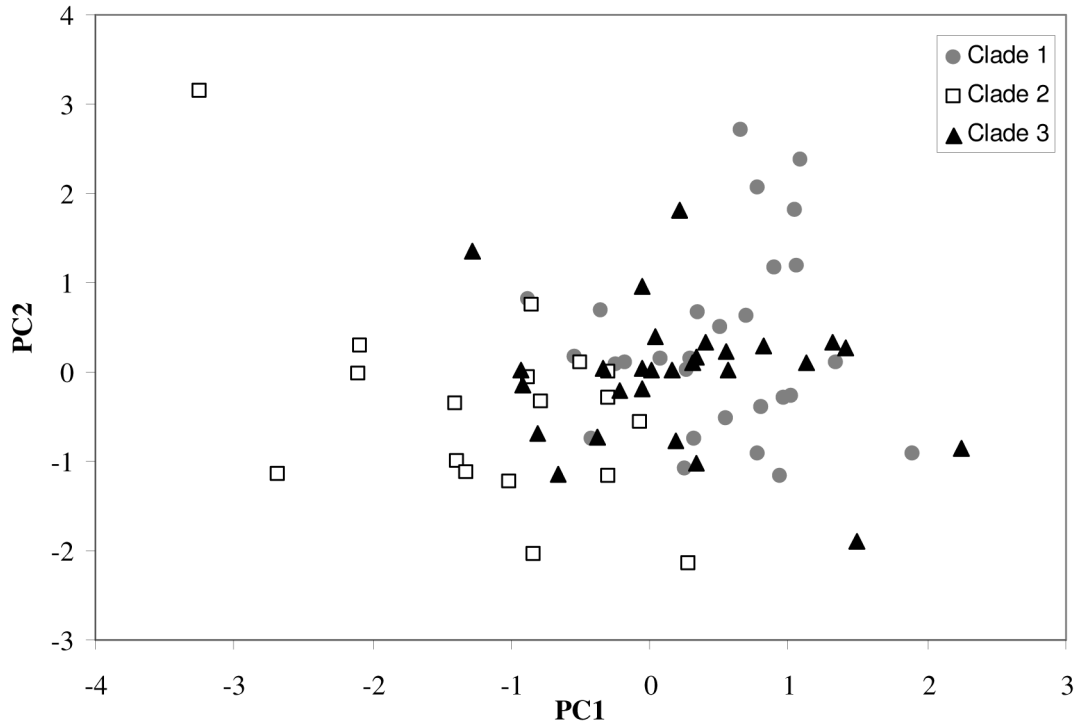

Figure 2-3. Bivariate plot of principal components derived six call characteristics.

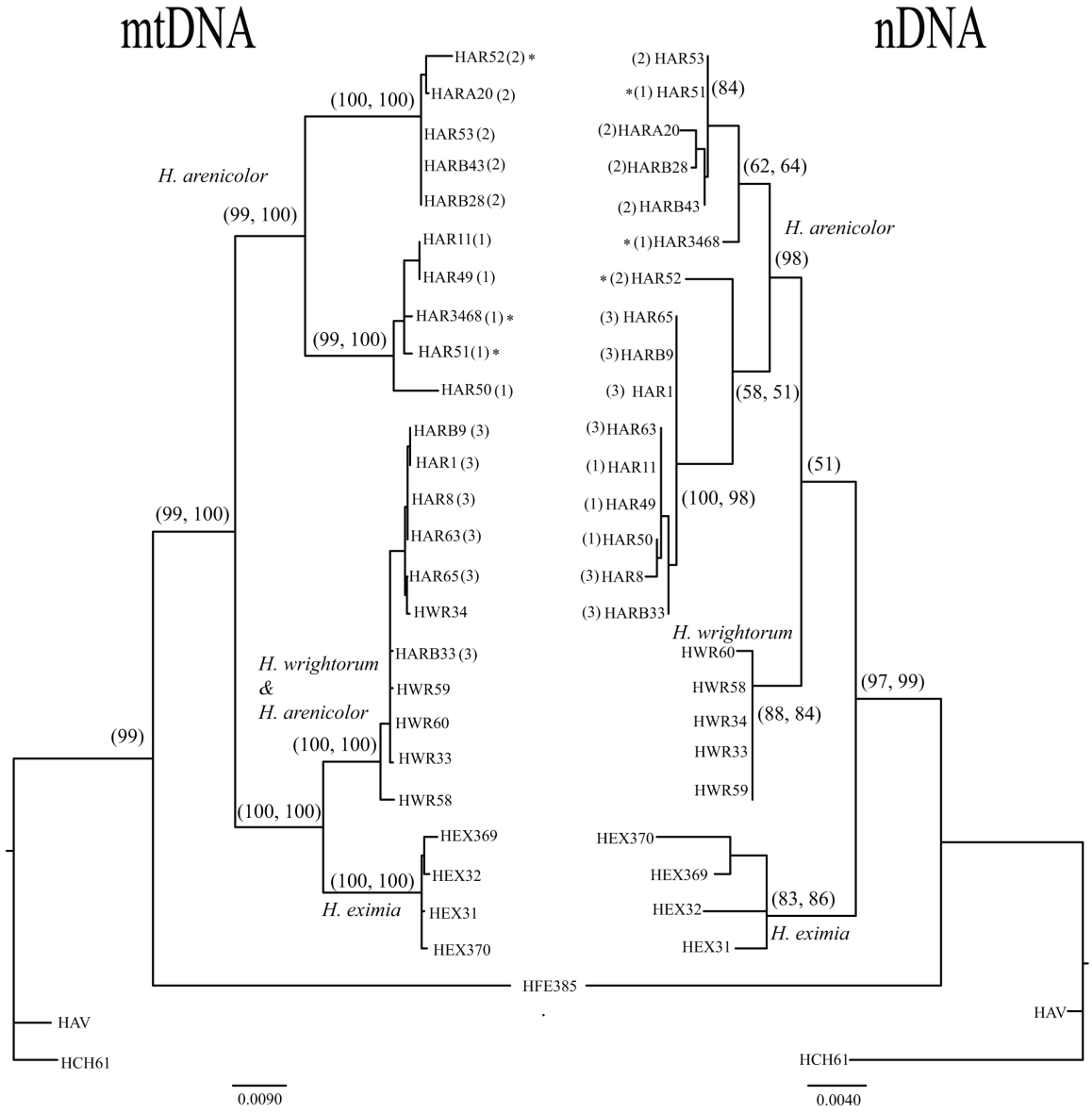
mtDNA
nDNA
HAR52 (2) *
HARA20 (2)
(100, 100)
HAR53 (2)
HARB43 (2)
H. arenicolor
HARB28 (2)
(99, 100)
HAR11 (1)
HAR49 (1)
HAR3468 (1) *
(99, 100)
HAR51 (1) *
HAR50 (1)
HARB9 (3)
HAR1 (3)
HAR8 (3)
(99, 100)
HAR63 (3)
HAR65 (3)
HWR34
H. wrightorum
&
H. arenicolor
HARB33 (3)
HWR59
HWR60
(99)
(100, 100)
HWR33
HWR58
(100, 100)
HEX369
HEX32
(100, 100)
H. eximia
HEX31
HEX370
HAV
HCH61
0.0090
HFE385
(2) HAR53
*(1) HAR51
(84)
(2)HARA20
(2)HARB28
(2) HARB43
*(1)HAR3468
(62, 64)
H. arenicolor
(98)
*(2) HAR52
(3) HAR65
(3)HARB9
(3) HAR1
(58, 51)
(3)HAR63
(1) HAR11
(1) HAR49
(1)HAR50
(3)HAR8
(3) HARB33
(100, 98)
(51)
H. wrightorum
HWR60
HWR58
HWR34
HWR33
HWR59
(88, 84)
(97, 99)
HEX370
HEX369
HEX32
(83, 86)
HEX31
H. eximia
HAV
HCH61
0.0040

Figure 2-4. Gene trees based on mitochondrial DNA sequences and nuclear sequences. Numbers in parentheses next to each *H. arenicolor* individual indicates which mitochondrial clade they belong to as defined by Barber (1999). Individuals marked with * are *H. arenicolor* individuals other than those from Clade 3 that changed position in the nuclear gene tree compared to the mtDNA topology. Values in parentheses at nodes indicate ML bootstrap values and MP bootstrap values, respectively. Nodes that only have one value do not include maximum parsimony support values as these nodes fell into a polytomy in the MP analysis.

CHAPTER 3

GEOGRAPHIC VARIATION IN MALE ADVERTISEMENT CALLS AND FEMALE PREFERENCE OF THE WIDE-RANGING CANYON TREEFROG, *HYLA ARENICOLOR*

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ABSTRACT

We surveyed geographic variation in male advertisement calls of the wide-ranging canyon treefrog, *Hyla arenicolor*. Using genetic lineages from a recent phylogeographic study to define our sampling, we found large call differences among geographically distant lineages. To test if these call differences are biologically relevant and could allow reproductive isolation of different lineages should they come into secondary contact, we assessed female preference in a lineage occurring in southern Utah and northwestern Arizona, USA. These females exhibited a strong preference for their own lineage's call type over the calls of two Mexican lineages but not calls from the geographically closest lineage. We also identified traits that female frogs probably use to discriminate between lineage-specific advertisement calls. Our behavioral results along with recent molecular studies estimating the phylogenetic relationships among lineages will allow for future work to address the evolutionary forces that have led to this biologically significant variation in male sexual signals.

INTRODUCTION

Conspicuous secondary sexual traits that play a role in mate attraction or assessment have long held the attention of biologists. The study of variation in these signals and receiver preferences reveals how these traits evolve, and how changes in signals and/or preferences might lead to behavioral isolation and ultimately speciation (Ryan et al., 1996). Behavioral isolation occurs when trait differences among species, or populations, decrease the attractiveness of heterospecific individuals (Coyne & Orr,

2004). This can result in assortative mating, reproductive isolation, reduced gene flow, and ultimately speciation (Dobzhansky, 1937). Variation in signal and preference arises by natural selection, sexual selection, genetic drift, or some combination of these factors (Mayr, 1963; Lande, 1981; West-Eberhard, 1983; Panhuis et al., 2001). The documentation of geographic variation in both signals and preferences is the first step in assessing how these traits evolve and contribute to behavioral isolation (Endler & Houde, 1995; Gray & Cade, 2000; Uy & Borgia, 2000; Pröhl et al., 2006). Such studies have not only provided insights into the mechanisms to diversification but have also led to the discovery of cryptic species (Lambert & Paterson, 1982; Shaw, 2000; Bickford et al., 2007; Lemmon et al., 2007).

Here we examine geographic variation in male advertisement calls and female preference in a treefrog, *Hyla arenicolor*. The canyon treefrog has a wide distribution, extending from the southwestern USA into south-central Mexico (Fig. 3-1). As in other North American hylid frogs, males produce advertisement calls to attract females for mating, and female preference for these calls can be easily assessed through playback experiments. Pierce (1968) reported the existence of distinctive advertisement calls in populations of canyon treefrogs in the Balsas Basin of south-central Mexico suggesting population divergence or even speciation. A recent investigation among the geographically neighboring USA lineages revealed little call differentiation, and showed that the differences in key acoustic properties known to affect female choice would probably be too small to promote assortative mating by lineage (Klymus et al., 2010). In this same study, phylogenetic relationships among these three mtDNA lineages as described by Barber (1999a) was found to be incongruent with nuclear sequence data due

to the past hybridization and mtDNA introgression with the sister species group *H. wrightorum/ H. eximia* (Klymus et al., 2010). In light of the new data, the authors showed that call differentiation better reflected nuclear phylogenetic relationships rather than relationships inferred from mtDNA sequence. Thus, the geographically and phylogenetically closest lineages (Grand Canyon – Barber's Clade 3 and the Colorado Plateau- Barber's Clade 1B) show the least amount of call divergence, a result that would be expected if drift, rather than selection, played a major role in population differentiation.

In this study we expand upon our original call analysis to include sampling throughout the species' range and to examine if call differentiation and female preference may lead to behavioral isolation among lineages. Our current study is based on a recent range-wide phylogeographic study of the canyon treefrog combining mitochondrial and nuclear sequence data (Bryson et al. 2010). Their phylogeographic lineages are used as the framework for our current call analysis: Colorado Plateau (Barber's Clade 1B); Grand Canyon (Barber's Clade 3); Sonoran Desert (Barber's Clade 2); Chihuahuan Desert (Barber's Clade 1A); Central Mexican Plateau; South West-Central Mexican Plateau; and the Balsas Basin. Assuming that the Mexican lineages are genetically more distant due to the effects of distance on gene flow, we expected random drift to lead to a higher call divergence among the geographically distant Mexican lineages relative to the USA lineages. We hypothesized that females from the USA lineage would show a preference for their own lineage's calls when presented with divergent call types of the Mexican lineages. We also tested the expectation that these females would not show a preference when presented with calls from one of the other USA lineages because of the

demonstrated similarity in the calls of these lineages (Klymus et al., 2010). By conducting tests with digitally edited calls, we also identified some of the acoustic differences responsible for between-clade call discrimination. Finally, since phylogenetic studies (Bryson et al., 2010; Klymus et al., 2010) inferred multiple episodes of past hybridization between *H. arenicolor* and *H. wrightorum/ H. eximia,* we tested for behavioral isolation via female preference between these two species groups.

METHODS

Sampling and Population Designation

We used Bryson et al.'s (2010) range-wide phylogeographic study to categorize our sample of recorded advertisement calls (Fig. 3-1). Within the USA, Barber's mitochondrial clades are hereafter referred to as the Colorado Plateau (CP) for Clade 1B, Grand Canyon (GC) for Clade 3, and Sonoran Desert (SD) for Clade 2. Within Mexico, samples are also designated by the phylogeographic lineage in which they were recorded. Although our sampling in the ranges of these lineages are limited, we are confident that the males whose calls were sampled in these regions belong to the respective phylogeographic lineages as outlined by Bryson et al. (2010). Our Guanajuato samples are in Bryson et al.'s Central Mexican Plateau range (CMP), and our Jalisco samples are from Bryson et al.'s Central Mexican Plateau-Southwest (CMP-SW). Our Balsas Basin samples from the state of Oaxaca correspond with Bryson et al.'s Balsas designation (BAL) (Fig. 3-1). Previously we included data from four males from Texas in our original call analysis; the geographic location of this population conforms to the hypothetical range of Barber's Clade 1A and Bryson et al.'s Chihuahuan Desert (CD)

lineage, which is most closely related to the Central Mexican Plateau lineage (2010). Removal of data from the Texas frogs had little influence on the average call trait values reported in our previous analysis (Klymus et al., 2010). Because of the small sample size of recordings from the Chihuahuan Desert (CD) lineage we consider them separately in this study. Furthermore, in our current study we restricted statistical analyses to calls from lineages with larger sample sizes: the three USA lineages (CP, GC, SD) and three Mexican lineages (CMP, CMP-SW and BAL). Our call sampling localities are listed in Table 3-1. Throughout the paper we refer to the lineages by their abbreviations listed above.

Recordings

We recorded 201 males in the field from southern Utah in the USA to the southern Mexican state of Oaxaca. Recordings were made using a Sony stereo cassette recorder (TC-D5M), a Tascam DAT recorder (DA-P1), or a solid-state Marantz digital recorder (PMD670). We recorded at least 10 consecutive calls by placing a Sennheiser directional microphone (ME-66) within 50-100 cm of the male frog. The calling male's deep body temperature was measured to the nearest 0.1° C with a Weber mercury quick-read cloacal thermometer. Calls recorded on tape cassettes were digitized at a sampling rate of 44 kHz using Sound Edit 16 v.2 (Macromedia, 1996).

Call Analysis

We analyzed five sequential advertisement calls from each male using the Raven 1.2.1 software package (Cornell Lab of Ornithology, 2003). Temporal properties were measured from oscillogram displays (Fig. 3-2a-c), and power spectra (Hamming window, FFT length = 1024 samples) were used to measure spectral properties (Fig. 3-2d).

Temporal traits measured included call duration, call period, pulse rate, pulse duration, inter-pulse interval, sub-pulse period, call duty cycle, and percentage pulse rise-time (Appendix B). The spectrum of the call is distinctly bimodal. We estimated the frequencies with greatest relative amplitude in the low- and high-frequency range and hereafter refer to these frequencies as the LFP (low-frequency peak) and HFP (high-frequency peak) respectively.

Call traits with a significant correlation with deep body temperature were temperature-corrected using a linear regression. These traits from the three Mexican populations were not temperature-corrected because the smaller sample sizes precluded estimation of reliable temperature-trait correlations. Because of this limitation, we could not compare the three Mexican populations with one another as they were recorded at widely different temperature ranges. Instead, we compared data from each Mexican lineage to that of the USA lineages, which were temperature corrected. The following comparisons were made: CMP to USA calls adjusted to 16 °C; calls from BAL to USA calls adjusted to 20 °C; and calls from CMP-SW to USA calls adjusted to 23 °C.

We analyzed call variation using R version 2.10 (R Foundation for Statistical Computing). For each male we estimated mean values of call traits from five calls. We then calculated grand means and standard deviations for each trait for the six lineages. All call traits were analyzed for departure from normal distribution. Call duration, call period and low frequency peak were log transformed to meet the assumptions of normality and homoscedacity. For each of the temperature-corrected traits, we used a one-way ANOVA and Tukey pairwise comparisons with Bonferonni correction to compare population means between the three USA lineages and the three Mexican

lineages. For traits that did not vary with temperature (percentage pulse rise-time and call duty cycle), we conducted the same analyses but simultaneously compared data from the six lineages. For comparisons of trait differences that we might expect to elicit female preference, we also express trait differences as percentages relative to the average of the USA lineages. For example, differences in pulse rate of 20% are needed to elicit preference for conspecific values in the gray treefrog (*H. chrysoscelis*) (Gerhardt, 2005).

Female Preference Tests

We tested whether female preference might lead to behavioral reproductive isolation among genetic lineages using phonotaxis experiments with female *Hyla arenicolor* from the CP and GC lineages. Because of their genetic and behavioral similarity (Klymus et al., 2010), we refer to the tested females as coming from a combined CP/GC lineage. We were unable to locate permanent, large populations of breeding treefrogs from the SD lineage in southern Arizona or from Mexican populations. We tested CP/GC females against the calls of the SD, CMP-SW and BAL lineages, along with calls in which we manipulated traits that showed the largest variability among lineages. Calls of the CMP type were not used as variation in these calls had not been measured at the time of the playback experiments.

Playback experiments were conducted during 2008-2010 at two field sites: Diamond Creek, Grand Canyon, Arizona, USA and Pine Creek, Zion National Park, Utah, USA. In the field, females were caught while in amplexus with males. They were then separated and placed in a portable playback arena. The arena consisted of a one by two meter enclosure made from wooden framed panels overlaid with black, cotton cloth. The arena was placed on a plastic tarp, and two speakers (L210 speaker system, Audio

and Digital Systems, inc. Wilmington, MA, USA) were placed outside of the arena facing inward, two meters apart from one another. The panels reduced the effects of wind and external visual stimuli. Test were conducted in the evening, only during quiet, gust free periods at a temperature range between 18°C and 21°C. Females were either released at the site of capture the following evening, or shipped back to the University of Missouri for further testing in the lab. Handling of animals complied with a protocol (#6546) approved by the Animal Care and Use Committee of the University of Missouri, Columbia.

In the lab, females were tested in a temperature controlled, semi-anechoic chamber. Because females do not respond to calls unless they are in breeding condition, we injected females with progesterone and prostaglandin, based on the protocol from Gordon and Gerhardt (2009).

In both the lab and the field, females were placed equidistant between the two speakers in an acoustically transparent holding cage. Calls were played for one minute before females were released. Calls were played at 85 dB SPL at one meter from each speaker. A response was reported if the female moved within ten centimeters of a speaker and stayed there for at least ten seconds. Stimuli were switched periodically between speakers to prevent side biases. For all the choice experiments, binomial exact tests were used to test significance of results.

Acoustic stimuli consisted of natural exemplars extracted from recordings using Adobe Audition 2.0 software (Adobe Systems, Inc.). To avoid a bias based on a particular exemplar, we used two different natural exemplars for each set of tests. Unless noted, sixteen females were tested in each set with eight females being tested with one

exemplar pair (e.g., recording of local male and recording of a male from another lineage/ species), and another eight females tested with a different exemplar pair.

Heterospecific test

Because previous work indicates that *H. arenicolor* and *H. wrightorum* hybridized in the past (Bryson et al. 2010, Klymus et al. 2010), we determined if females preferred the conspecific call over that of the sister species, *H. wrightorum*. Each test consisted of a natural *H. arenicolor* call from the USA and a natural *H. wrightorum* call from the USA. We did not find sufficient numbers of *H. wrightorum* females to warrant testing.

Conspecific tests

To examine preferences for lineage specific call types, females from the CP/GC lineage were presented with three tests: SD vs. CP/GC lineage calls, CMP-SW vs. CP/GC lineage calls, and BAL vs. CP/GC lineage calls. Stimuli were presented in an alternating fashion to avoid acoustic overlap; however, because of large differences in call duration and period between CP/GC and BAL calls, there was no way to present alternative stimuli without overlap. Such overlap created a leader/follower relationship between stimuli that could influence female preference. Accordingly, we constructed two experimental sets, with 16 females tested in each set. In one set the CP/GC call was in the leading position and in the other set, the BAL call was in the leading position (Fig. 3-3).

Pulse rate tests

To test the effects of different pulse rates on CP/GC females, we edited natural exemplars with Adobe Audition. We extracted a pulse from the middle of a CP/GC call and repeatedly inserted it along with an appropriate silent inter-pulse interval to construct a new call. Specifically, these calls had the average CP/GC call duration but either the

slower BAL pulse rate or the faster CMP-SW pulse rate, respectively (Fig. 3-4). As before, two different natural exemplars were used for each set. These stimuli each had the average CP/GC call duration and were presented to the females in an alternating fashion to prevent acoustic overlap.

Call duration/ call period tests

To assess the behavioral significance of the distinctive call period and call duration of the BAL lineage, we used Adobe Audition to alter the duration of representative calls of the CP/GC males to match the average duration of BAL calls. These short duration calls were then inserted so that the call period was representative of that of the BAL males (Fig. 3-5). To avoid a leader/follower bias, stimuli were presented in no-choice experimental design (Fig. 3-5).

In this design, females were first given two control stimuli (the normal CP/GC call) followed by the test stimuli (the CP/GC call shortened and repeated to the average BAL values). A third control (the normal CP/GC call) then followed the test stimulus. Within a testing session, a female was placed in the center and given silence for one minute, followed by a stimulus played for one minute before the female was released. After her response, she would be placed back in the center of the arena. This procedure was repeated for all four stimuli (control, control, test, control) in one female's session. Response time was recorded for each stimulus. Phonotaxis scores were calculated as a ratio of the female's average response time to the 2nd and 3rd controls to her response time to the test stimulus. Females that either did not move from their release point after ten minutes, or who left the arena without approaching the speaker, but did respond to the last control stimulus, were given a phonotaxis score of zero. Ten different females were

tested in one of two conditions: either both the control (CP/GC) and test (BAL) stimuli had an equal call duty cycle or the stimuli had unequal call duty cycles.

RESULTS

Call Analysis

Call traits varied the most between USA (CP/GC/SD) and Mexican lineages, with relative percentage differences of some traits exceeding 80 % (Table 3-5). This differs markedly from comparisons among USA lineages, in which traits varied less than 18 % (Table 3-5) and trait variation broadly overlapped among the USA lineages (Fig. 3-6). For a graphical representation of the percentage differences, we highlight the relative differences for traits known to be important for female preference in other hylids, namely frequency peak and pulse rate, as well as call duration and call period (Gerhardt & Huber, 2002) (Fig. 3-7). Results from ANOVAs and post-hoc pairwise comparisons revealed significant differences between the three USA lineages and each Mexican lineage for all traits ($p<0.05$, Table 3-2, Table 3-3, Table 3-4). Comparisons among the three USA lineages also show significant differences in all traits, except inter-pulse interval.

Among USA lineages

Results of our univariate call analyses among the three USA lineages parallels that of our earlier multivariate analysis (Klymus et al., 2010), with the SD clade (Clade 2) showing the greatest divergence vis-à-vis other USA lineages (Table 3-2, Table 3-3, Table 3-4) (Fig. 3-6). The SD males produced shorter calls, with a shorter call period, longer pulse duration, shorter sub-pulse period, and lower pulse rate than the calls of the other two lineages. Spectrally, the SD lineage calls have higher frequency peaks in both

the low and high frequency ranges than calls of the other lineages. The CP lineage had significantly longer calls with a higher pulse rate than the other two USA lineages. The GC lineage showed a lower percentage pulse rise-time and a lower call duty cycle than the other two USA lineages.

Despite the statistically significant differences revealed by the ANOVA, the percentage differences among the three USA lineages for all traits measured are less than 18% (Table 3-5).

BAL and USA

As was expected from Pierce's (1968) study the calls from the BAL lineage are shorter in duration and shorter in call period. Furthermore, we found that they had a lower pulse rate and longer inter-pulse interval with percentage differences over 30% (Table 3-5, Fig. 3-7). Balsas calls have a sub-pulse period that is not significantly different from USA lineages, nor does their high frequency peak differ significantly. The low frequency peak of BAL lineage calls are significantly higher than those of the CP and GC lineages, but not different from those of the SD clade. For the temperature-uncorrelated traits, the BAL lineage had a significantly lower percentage pulse rise-time and higher call duty cycle among all six lineages.

CMP-SW and USA

Most traits exhibit percentage differences between CMP-SW and USA populations that are comparable to the average difference observed among USA lineages (Table 3-5). However, pulse rate, inter-pulse interval, and high frequency peak exhibited the largest percentage differences (> 20%) relative to USA populations (Table 3-5, Fig. 3-7).

These calls have a significantly higher pulse rate and shorter inter-pulse intervals than all the USA lineages. Pulse duration is generally shorter than that of the USA lineages, but it is only significantly shorter in comparison to the SD clade. The sub-pulse period is also shorter than that of the three USA lineages, but not significantly so from the SD lineage. Percentage pulse rise-time and call duty cycle are similar to the values in USA calls. Regarding spectral components, calls from the CMP-SW clade have higher frequency peaks than those of the GC or CP lineages, but these are not significantly different from the SD lineage.

CMP and USA

As in comparison with CMP-SW calls, the relative percentage difference between CMP and USA populations in most traits was comparable to the difference observed among USA lineages. Similar to the CMP-SW calls, CMP pulse rate and inter-pulse interval also have percentage differences larger than 30%, considerably larger than differences observed among USA lineages (Table 3-5, Figure 3-7).

Calls of males from the CMP lineage have a duration and period within the variation range of the three USA lineages. Pulse rate is higher and pulse duration and inter-pulse intervals are shorter than calls of the USA lineages; CMP calls also had shorter pulse duration and shorter inter-pulse intervals. Sub-pulse period was not significantly different from that of USA lineages, and both frequency peaks are also within the USA lineages' range of variation. For traits uncorrelated with temperature, calls of the CMP lineage had a significantly higher percentage pulse rise-time than all lineages except the SD lineage. Call duty cycle was comparable to the CMP-SW, CP and

SD lineages; however, it was higher compared to the GC lineage and lower relative to the BAL lineage.

Texas (Chihuahuan Desert- CD) and USA

Because of the small sample size of Texas population we did not conduct statistical comparisons, but we show percentage differences between the Texas (CD) population and other lineages (Table 3-5). The largest percentage differences, relative to variation among USA populations, were in pulse rate and correlated traits (ie. inter-pulse interval). These traits have percentage differences similar to those observed for the CMP-SW and CMP comparisons with USA populations (Table 3-5), and these differences are in the same direction: faster pulse rates and shorter inter-pulse intervals.

Female Preference

Heterospecific

Female *H. arenicolor* showed a strong preference for their owns species' call over the call of the heterospecific, *H. wrightorum*, with all 16 females choosing the *H.arenicolor* call in the lab (exact binomial test, $P<0.001$), and 15 out of 16 females choosing the *H. arenicolor* call in the field (exact binomial test, $P<0.001$).

Conspecific

Previously we found small but statistically significant call differences between CP/GC lineage and the SD lineage that we predicted to be biologically insignificant (Klymus et al., 2010). As predicted, female *H. arenicolor* from the CP/GC lineage did not prefer their own lineage's call to the calls of the SD lineage. In both the lab and the field, females chose the SD call over their own population's call roughly half the time. In the lab, 9 out of 16 females chose the CP/GC call over the SD call (exact binomial test, p

= 0.8036). In the field, 7 out of 16 females chose the CP/GC call over the SD call (exact binomial test, p = 0.8036).

However, CP/GC females strongly preferred their own lineage's call over either the BAL or CMP-SW calls in both the lab and the field. In the lab, 16 females were given the choice between a CP/GC leading and BAL following natural call (Fig. 3-3). All 16 females chose the CP/GC call (exact binomial test, $p < 0.001$). Similarly, 16 females were given the choice between the two calls in which the BAL call led. In this set, 15 of the females chose the CP/GC call (exact binomial test, $p < 0.001$). In the field, a total of 20 females were again tested against natural calls in which only the BAL call led. In the field all 20 females chose the natural CP/GC call, even though it was in the following position (exact binomial test, $p < 0.001$). With respect to the CMP-SW call, 13 out of 16 females tested in the lab chose the CP/GC call type over the CMP-SW call (exact binomial test, $p < 0.05$). Similarly, in the field 15 out of the 16 females chose the CP/GC call type (exact binomial test, $p < 0.001$).

In summary, CP/GC females showed no preference for their lineage's own call over the call type from the SD; however, females strongly preferred their lineage's call type over those calls from the geographically distant BAL and CMP-SW lineages.

Pulse rate

Because CP/GC females preferred their lineage's call type over that of the BAL or CMP-SW lineages, we tested our hypothesis that the large difference in pulse rate was sufficient to isolate frogs in these lineages. For the BAL call type, females were given the choice between two stimuli, one with the average pulse rate and pulse duration for the CP/GC lineage and the other stimuli with the average BAL values for pulse rate and

pulse duration. In this test the pulse duty cycle was higher in the CP/GC call at 65% compared to 55% for the BAL call. In the lab, 13 out of 16 females chose the CP/GC pulse rate stimulus (exact binomial test, $p<0.05$). In field conditions, 15 out of 16 females chose the CP/GC pulse rate over that of the BAL lineage (exact binomial test, $p < 0.001$).

Females from USA lineages were also tested against calls with the average CMP-SW pulse rate and pulse duration. In the lab 15 out of 16 females showed a preference for the CP/GC pulse rate stimulus (exact binomial test, $p < 0.001$). In the field, females also chose their lineage's pulse rate over the faster pulse rate of the CMP-SW lineage. A total of 13 out of 16 females chose the CP/GC pulse rate over that of the CMP-SW lineage (exact binomial test, $p < 0.05$).

Call duration/ call period

For the no-choice tests, a phonotaxis score of greater than one means females moved to the test (BAL) duration and rate stimulus faster than they did to the CP/GC control. Values less than one signify they moved more slowly to the BAL stimulus. In the field, the average phonotaxis score for the unequal call duty cycle was 0.33 (ten females tested, five responded to test stimulus), and for the equal call duty cycle the mean phonotaxis score was 0.24 (ten females tested, three responded to test stimulus). According to Bush and Schul (2002) values below 0.40 are relatively unattractive. Only one female responded slightly faster to the test stimulus than to the average control, with 11 of the 20 females not responding to the test stimulus at all and thus had a phonotaxis score of zero. Similarly, a majority of females in the lab did not respond to the BAL test stimulus. Interestingly, most of these no responses occurred in the unequal-call duty cycle

tests, in which the BAL stimuli even had more acoustic energy than the CP/GC control stimulus. The mean phonotaxis score for the unequal call duty cycle was 0.16 (ten females tested, two responded to test stimulus). For the equal-call duty cycle, ten females were tested, and only eight responded to the test stimulus; the mean score was 0.53. The lab equal-call duty cycle test was the only test in which the average phonotaxis score was above 0.40.

Overall, field and lab tests clearly show that females are less attracted to the BAL stimulus than to calls of CP/GC males. The average phonotaxis scores for the equal and unequal call duty cycle tests, however, appear to show slightly opposite trends between the lab and the field. This difference in average scores between lab and field, however, is not statistically significant (t-test, $p > 0.1$).

DISCUSSION

Our study reveals range-wide geographic variation in male advertisement calls of the canyon treefrog, some of which is biologically relevant and could potentially lead to reproductive isolation among the geographically more distant lineages. Variation exists among the three USA lineages (GC, CP, SD); however, these differences are small. More importantly, we confirm the expectation that call differentiation among the three USA lineages is not biologically significant. Females from the CP/GC lineages did not have a preference for their own lineage's call over the SD call type, suggesting that the existing call differences would not be sufficient to prevent these females from mating with SD males if they come into contact. Genetic data suggests no gene flow currently exists between them (Barber, 1999b); however as they are geographically near one another,

secondary contact is a distinct possibility should the current xeric conditions now separating them become more mesic in the future.

As we predicted, the largest call differences exist between USA lineages and Mexican lineages (CMP-SW, CMP, and BAL), and these differences have the potential to serve as effective behavioral isolating mechanisms. Frequency affects female preference in several anuran species, but the low percentage differences observed among lineages in our study suggests that frequency differences are not likely to be used by females (Fig. 3-7), especially as frequency preferences can be easily reversed by changes in sound intensity (Gerhardt & Huber, 2002). All three Mexican lineages show large percentage differences in pulse rate and a correlated trait, inter-pulse interval (Table 3-5, Fig. 3-7). In particular, pulse rate differences in the three Mexican lineages well exceeds the 20% difference shown to be sufficient to mediate call preferences in other hylid species (Gerhardt & Huber, 2002; Gerhardt, 2005). Our results show that females from the CP/GC lineage discriminate against calls with the higher pulse rate of CMP-SW males and the lower pulse rate of BAL males. The advertisement calls from the BAL lineage also show large percentage differences in call duration (56.58%) and call period (71.27%) compared to USA lineages (Table 3-5, Fig. 3-7). No-choice tests suggest that females are also able to use this difference in call duration and call period to discriminate in favor of lineage specific calls regardless of pulse rate differences. An intriguing finding from the call analysis is that BAL lineage calls have a high frequency peak that is not significantly different from any of the three USA lineages, and their low frequency peak is slightly higher than those of the USA. The BAL males, however, are much larger in size (53.1 mm snout vent length) than other *H. arenicolor* males (40.0 – 48.0 mm

snout vent length) (Pierce ,1968; Duellman, 1970). This is interesting because in many frog species frequency is inversely correlated with body size (Gerhardt & Huber, 2002- these authors also point out a few exceptions to this generalization). Thus we might expect the larger BAL males to have lower frequency peaks compared to the relatively smaller males of other lineages.

Although USA females from the GC/CP lineage show a strong preference for the call type of their own population over those of the Mexican lineages, female preference needs to be assessed among the other lineages. Of particular interest would be investigating whether selection has played a role in driving call divergence along the contact zones among the three Mexican lineages, as the CMP and CMP-SW show the largest degree of call divergence (pulse rate, call period and call duration) relative to the BAL lineage. The phylogeographic study by Bryson et al. (2010) shows that the BAL and CMP-SW lineage occur within 100 km of one another, which suggests that these lineages are likely to come into contact with one another. Further study may identify the relative importance of factors isolating these lineages (e.g. behavioral, geographical, ecological). Finally, although we only analyzed four males calls from Texas (belonging to the CD lineage), pulse rate and the correlated inter-pulse interval show a larger percentage difference vis-a-vis USA populations than is seen among USA populations. Furthermore, these differences are in the same direction as those observed in the CMP-SW and CMP lineages. In fact, Pierce (1968) described two Texas populations (n=23 and n=12) as having higher temperature corrected pulse rate values than those of other USA populations, results congruent with our own. Although suggestive, more sampling is

needed in order to assess whether the calls of the CD lineage are more similar to calls of the other USA lineages or more similar to the CMP and CMP-SW lineages.

Recent molecular studies have shown that introgressive hybridization occurred between *H. arenicolor* lineages and species from the sister group *H. wrightorum/ eximia* (Bryson et al., 2010; Klymus et al., 2010). Besides gross morphological differences, advertisement calls from this group are qualitatively very different from calls of canyon treefrogs including those of the BAL lineage (Duellman, 1970; Gergus et al., 2004) (Fig. 3-2). Females of either species would likely show a strong preference for their conspecific call. In fact, our behavioral results suggest that these species are likely to be behaviorally isolated, as female canyon treefrogs strongly prefer the calls of *H. arenicolor* to those of *H. wrightorum* male. However, the occurrence of unidirectional introgression suggests that mismating was primarily between *H. wrightorum* females and *H. arenicolor* males (Bryson et al., 2010; Klymus et al., 2010). Thus, species discrimination needs to be tested in females of *H. wrightorum*. If *H. wrightorum* females do not show a preference, this may partly explain the observed unidirectional introgression. Nevertheless, even if behavioral pre-mating isolation exists between both species, mismatings and introgression is still likely to occur as anuran males are often indiscriminate when choosing a mate and will clasp any frog that comes near them (Blair, 1958). Furthermore, mismatings between otherwise behaviorally isolated species is well documented, especially in disturbed environments (Lamb & Avise, 1986; Lemmon et al., 2007). Such mismatings can lead to mitochondrial introgression which is believed to occur more readily than introgression for nuclear alleles (Funk & Omland, 2003). Chan and Levin (2005) developed mathematical models that showed the introgression of

maternally inherited cytoplasmic markers (mtDNA) can occur more rapidly than the introgression of other genes even with prezygotic reproductive barriers in place. This rapid introgression may be especially likely when one species is more abundant that the other in a particular breeding location. Females of the less abundant species are more likely to encounter and be amplexed by males of the more abundant species. Such a scenario is realistic and supported by field observations in Arizona (Gerhardt, pers. obs.) in which mixed breeding choruses are dominated by one species, although no mixed species mating pairs or hybrid offspring have been reported. Further field studies on female preference in both species could confirm the importance of frequency-dependent mate choice in this system.

Upon Pierce's (1968) description of call differentiation among the Balsas Basin canyon treefrogs, he suggested this population may be a distinct species. However, he felt that further behavioral research would be needed to confirm this conclusion and thus considered the Balsas (BAL) group as a divergent population. General morphological and call similarities would suggest this group is sister to other *H. arenicolor*. However, recent phylogenetic assessment questions this lineage's placement within *H. arenicolor* (Bryson et al., 2010). Using two nuclear introns Bryson et al.'s study placed this group outside of all other *H. arenicolor* and *H. wrightorum/ H. eximia* lineages, suggesting that *H. arenicolor* is a paraphyletic group (2010). Further molecular phylogenetic analyses may be able to better resolve the relationships of *H. arenicolor*, the Balsas *H. arenicolor* and *H. wrightorum/ eximia* lineages. Regardless of the lack of phylogenetic resolution, the degree of call differentiation and female discrimination support Pierce's original hypothesis that the Balsas Basin lineage represents a distinct species.

Overall, our results indicate striking differences in pulse rate, call duration and call period among lineages of the canyon treefrog. Females can use these differences to discriminate against calls, and females of one lineage (CP/GC) show significant preference for their conspecific lineage call relative to calls of two of the Mexican lineages. Now that we have documented geographic variation in call traits, the next question to ask is how this variation arose. Several studies have used molecular techniques to address the varying roles that natural selection, sexual selection and drift may play in behavioral isolation. For instance, using coalescent simulations with mtDNA sequence and signal data, Masta and Maddison (2002) and Boul et al. (2006) found evidence for sexual selection to have driven signal divergence in spider and frog species, respectively. By using partial Mantel tests to compare genetic distance, acoustic distance and ecological distances, mating song divergence in thrushes is thought to be more strongly affected by natural selection via acoustic transmission rather than genetic drift (Ruegg et al., 2006). Such methods would be the next step to apply in our study system; however, we need increased call sampling throughout the Mexican lineages (and the Chihuahua Desert Lineage) to allow for temperature correction of call traits before calculating call distances. Furthermore, quickly evolving neutral nuclear markers are needed to calculate genetic distances. Due to introgression of the *H. wrightorum/ eximia* mitochondrial genome into two canyon treefrog lineages (Bryson et al., 2010; Klymus et al., 2010), mitochondrial sequence data will not give accurate genetic distances. Future studies integrating these techniques and addressing the possible role of geographic barriers to gene flow may be able to elucidate more clearly the forces that have driven

this divergence in advertisement calls, and how this divergence may influence the evolutionary trajectories of these lineages.

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Table 3-1. Sampling localities including the symbols for each location used in Fig. 3-1. The lineage refers to the phylogeographic lineage (Bryson et al., 2010) for each sampling locality.

Location	Map Symbol	Lineage	Latitude	Longitude
Davis Mountains, TX, USA	DM	CD	30.700	-103.500
Seven Springs, Mogollon Rim, AZ, USA	SS	CP	33.967	-111.865
Reynold's Creek, Sierra Ancha Mountains, AZ, USA	SA	CP	33.874	-110.992
Lower Workman Creek, Sierra Ancha Mountains, AZ, USA	SA	CP	33.846	-110.972
Parker Canyon, Sierra Ancha Mountains, AZ, USA	SA	CP	33.798	-110.967
Jones's Crossing, Mogollon Rim, AZ, USA	JC	CP	34.529	-111.285
Sycamore Creek, Mogollon Rim, AZ, USA	SY	CP	33.867	-111.465
Pine Creek, Zion National Park, UT, USA	ZNP	CP	37.217	-112.974
Bright Angel, Grand Canyon, AZ, USA	BA	GC	36.099	-112.095
Diamond Creek, Grand Canyon, AZ, USA	DC	GC	35.766	-113.373
Peach Springs, Grand Canyon, AZ, USA	DC	GC	35.586	-113.435
Spencer Canyon, Grand Canyon, AZ, USA	SP	GC	35.824	-113.648
Cave Canyon, Grand Canyon, AZ, USA	CC	GC	36.096	-113.921
Tanque Verde, Santa Catalina Mountains, AZ, USA	SB	SD	32.252	-110.737
Sabino Canyon, Santa Catalina Mountains, AZ, USA	SB	SD	32.317	-110.818
Jacobson's Creek, Pinaleños Mountains, AZ, USA	PM	SD	32.667	-109.803
Box Canyon, Santa Rita Mountains, AZ, USA	SR	SD	31.798	-110.777
Madera Canyon, Santa Rita Mountains, AZ, USA	SR	SD	31.735	-110.883
Scotia Canyon, Huachuca Mountains, AZ, USA	SC	SD	31.465	-110.462
Ruby Road, Tumacacori Mountains, AZ, USA	RR	SD	31.397	-111.139
Huajuapan de Leon, Oaxaca, MX	BAL	BAL	17.805	-97.781
Bosque de La Primavera, Jalisco, MX	CMP-SW	CMP-SW	20.596	-103.639
Jocotepec, Jalisco, MX	CMP-SW	CMP-SW	20.309	-103.445
Cerro Colorado, Guanajuato, MX	CMP	CMP	20.232	-100.347

Table 3-2. ANOVA results of all call trait comparisons among lineages. Comparisons among the three USA lineages corrected at 18° C are included for easy reference to results from our previous study (Klymus et al., 2010).

Trait	Temperature (°C)	Populations	N	ANOVA
High Frequency Peak	23	CMP-SW US-CP US-GC US-SD	23 45 69 36	F(3,169) = 26.43 p < 0.01*
	20	BAL US-CP US-GC US-SD	8 45 69 36	F(3, 154) = 12.03 p < 0.01*Ŧ
	16	CMP US-CP US-GC US-SD	12 45 69 36	F(3, 158) = 15.53 p < 0.01*
	18	US-CP US-GC US-SD	45 69 36	F(2,147) = 17.22 p < 0.01*
Low Frequency Peak (log)	23	CMP-SW US-CP US-GC US-SD	23 45 69 36	F (3, 169) = 16.20 p < 0.01*
	20	BAL US-CP US-GC US-SD	8 45 69 36	F (3, 154) = 17.71 p < 0.01*
	16	CMP US-CP US-GC US-SD	12 45 36 69	F(3, 158) = 14.52 p < 0.01*Ŧ
	18	US-CP US-GC US-SD	45 36 69	F(2,147) = 20.83 p < 0.01*
Call duration (log)	23	CMP-SW US-CP US-GC US-SD	23 40 71 33	F(3,163) = 11.06 p < 0.01*
	20	BAL US-CP US-GC US-SD	8 40 71 33	F(3, 148) = 42.92 p < 0.01*
	16	CMP US-CP US-GC US-SD	12 40 71 33	F(3,152) = 11.94 p < 0.01*
	18	US-CP US-GC US-SD	40 71 33	F(2,141) = 16.04 p < 0.01*

Table 3-2. Continued

Trait	Temperature (°C)	Populations	N	ANOVA
Call Period (log)	23	CMP-SW US-CP US-GC US-SD	22 37 59 24	F (3, 138) = 9.79 p < 0.01*Ŧ
	20	BAL US-CP US-GC US-SD	8 37 59 24	F(3,124) = 76.29 p < 0.01*
	16	CMP US-CP US-GC US-SD	11 37 59 24	F(3,127) = 10.14 p < 0.01*
	18	US-CP US-GC US-SD	37 59 24	F(2,117) = 12.60 p < 0.01*
Pulse Rate	23	CMP-SW US-CP US-GC US-SD	23 44 71 38	F(3, 172) = 231.39 p < 0.01*
	20	BAL US-CP US-GC US-SD	8 44 71 38	F (3, 157) = 107.51 p < 0.01*
	16	CMP US-CP US-GC US-SD	12 44 71 38	F(3, 161)= 63.94 p < 0.01*
	18	US-CP US-GC US-SD	44 71 38	F(2, 150) = 17.05 p < 0.01*
Pulse Duration	23	CMP-SW US-CP US-GC US-SD	23 41 63 32	F(3, 155) = 8.03 p < 0.01*
	20	BAL US-CP US-GC US-SD	8 41 63 32	F(3, 140) = 24.14 p < 0.01*
	16	CMP US-CP US-GC US-SD	12 41 63 32	F(3, 144) = 10.95 p < 0.01*
	18	US-CP US-GC US-SD	41 63 32	F(2,133) = 7.48 p < 0.01*

Table 3-2. Continued

Trait	Temperature (°C)	Populations	N	ANOVA
Inter-Pulse Interval	23	CMP-SW US-CP US-GC US-SD	23 42 63 31	F(3, 155) = 10.01 p < 0.01*
	20	BAL US-CP US-GC US-SD	8 42 63 31	F(3, 140) = 43.80 p < 0.01*
	16	CMP US-CP US-GC US-SD	12 42 63 31	F(3, 144) = 21.75 p < 0.01*
	18	US-CP US-GC US-SD	42 63 31	F(2,133) = 2.26 p = 0.1082
Sub-Pulse Period	23	CMP-SW US-CP US-GC US-SD	23 42 62 29	F(3, 152) = 7.61 p < 0.01*
	20	BAL US-CP US-GC US-SD	8 42 62 29	F(3, 137) = 4.02 p < 0.01*
	16	CMP US-CP US-GC US-SD	12 42 62 29	F (3, 141) = 4.79 p < 0.01*
	18	US-CP US-GC US-SD	42 62 29	F(2, 130) = 5.73 p < 0.01*
Percentage Pulse Rise-time		CMP-SW BAL CMP US-CP US-GC US-SD	23 8 12 42 65 28	F(5,172) = 11.64 p < 0.01*
Call Duty Cycle		CMP-SW BAL CMP US-CP US-GC US-SD	22 8 11 40 62 29	F(5,166)=18.34 p < 0.01*

* statistically significant differences
Ŧ significant among the US populations, not with the Mexican population

Table 3-3. Results of Tukey post-hoc comparisons with Bonferroni corrections for all pairwise comparisons of temperature corrected call traits.
Pairwise comparisons were not conducted if ANOVA was non-significant (-------).

High Frequency Peak				Low Frequency Peak		
	US-CP	US-GC	US-SD	US-CP	US-GC	US-SD
US-GC	p=0.68			p=0.22		
US-SD	p < 0.01*	p < 0.01*		p < 0.01*	p < 0.01*	
CMP-SW	p < 0.01*	p < 0.01*	p=0.10	p < 0.01*	p < 0.01*	p=1.00
BAL	p=0.69	p=1.00	p=0.57	p < 0.01*	p < 0.01*	p=1.00
CMP	p < 0.01*	p < 0.01*	p=1.00	p=0.35	p=1.00	p=0.09
Call Duration				Call Period		
US-GC	p < 0.01*			p=1.00		
US-SD	p < 0.01*	p=1.00		p < 0.01*	p < 0.01*	
CMP-SW	p < 0.05 *	p=1.00	p=0.54	p=0.40	p=0.08	p=0.39
BAL	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*
CMP	p < 0.01*	p=1.00	p=1.00	p < 0.05 *	p < 0.05 *	p=1.00
Pulse Rate				Pulse Duration		
US-GC	p < 0.01*			p=1.00		
US-SD	p < 0.01*	p < 0.05 *		p < 0.05 *	p < 0.01*	
CMP-SW	p < 0.01*	p < 0.01*	p < 0.01*	p=0.30	p=0.89	p < 0.01*
BAL	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*
CMP	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.05 *	p < 0.01*
Inter-Pulse Interval				Sub-Pulse Period		
US-GC	----------			p=0.72		
US-SD	----------	----------		p < 0.05 *	p < 0.01*	
CMP-SW	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	p=1.00
BAL	p < 0.01*	p < 0.01*	p < 0.01*	p=1.00	p=1.00	p=0.53
CMP	p < 0.01*	p < 0.01*	p < 0.01*	p=0.12	p=0.55	p=1.00

Table 3-4. Results of Tukey post-hoc comparisons with Bonferroni corrections for all pairwise comparisons of non-temperature corrected call traits.

Percentage Rise-time					
	US-CP	US-GC	US-SD	CMP-SW	BAL
US-GC	p=0.17				
US-SD	p=1.00	p < 0.01*			
CMP-SW	p=1.00	p=1.00	p=1.00		
BAL	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	
CMP	p < 0.05*	p < 0.01*	p=1.00	p < 0.05*	p < 0.01*
Call Duty Cycle					
	US-CP	US-GC	US-SD	CMP-SW	BAL
US-GC	p < 0.01*				
US-SD	p=1.00	p < 0.01*			
CMP-SW	p=0.85	p=0.23	p=1.00		
BAL	p < 0.01*	p < 0.01*	p < 0.01*	p < 0.01*	
CMP	p=1.00	p < 0.05*	p=1.00	p =1.00	p < 0.01*

Table 3-5. Percentage differences in temperature corrected call traits relative to USA lineages (CP /GC/ SD).
The first row is the percentage difference observed among the three US lineages. Standard deviations are in parentheses. The number of individuals used to calculate these differences are the same as in Table 3-2, note the CD lineage only has 2 or 4 individuals.

	High Frequency Peak	Low Frequency Peak	Call Duration	Call Period	Pulse Rate	Pulse Duration	Inter-Pulse Interval	Sub-Pulse Period	Percentage Pulse Rise-Time	Call Duty Cycle
	US	US	US	US	US	US	US	US	US	US
US	11.27% (9.80)	8.90% (7.51)	13.69% (16.30)	17.36% (16.27)	5.28 % (4.17)	10.80% (8.78)	13.62% (10.66)	7.70% (6.88)	6.63% (5.14)	17.43% (12.99)
CMP-SW	20.22% (8.14)	8.80% (10.14)	20.42% (16.68)	21.30% (16.93)	30.67% (8.91)	11.69% (7.78)	29.62% (13.39)	8.83% (8.56)	5.33% (4.18)	16.05% (13.43)
BAL	5.12% (2.36)	10.92% (5.52)	56.58% (5.81)	71.27% (5.44)	34.93% (9.74)	45.40% (30.31)	86.37% (32.23)	3.97% (3.43)	14.07% (9.66)	51.78% (26.23)
CMP	13.32% (6.78)	5.17% (3.75)	11.83% (10.01)	21.04% (9.74)	30.71% (15.49)	14.64% (8.31)	34.55% (12.58)	7.48% (3.97)	9.68% (7.52)	16.92% (10.43)
CD	9.72% (9.78)	3.72% (3.35)	24.68% (25.01)	16.22% (1.09)	26.10% (13.85)	12.11% (4.73)	30.77% (7.76)	8.06% (3.19)	7.48% (4.02)	20.92% (7.77)

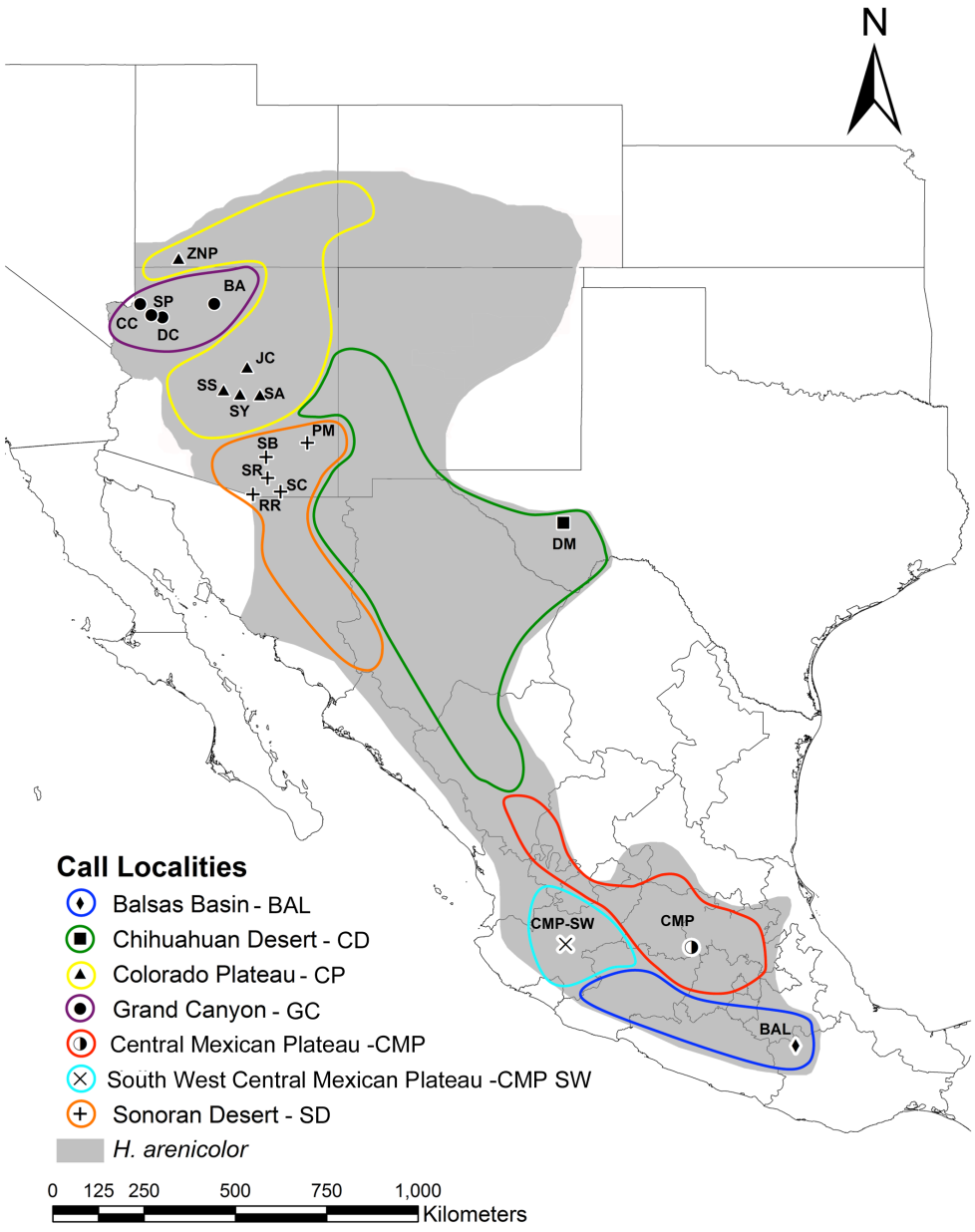


Figure 3-1. Phylogeographic lineages (Bryson et al., 2010) are outlined on the map. Points represent call sampling within these regions: symbols represent the lineage and sampling site, and letters refer to the specific sampling locality as referenced in Table 3-1.

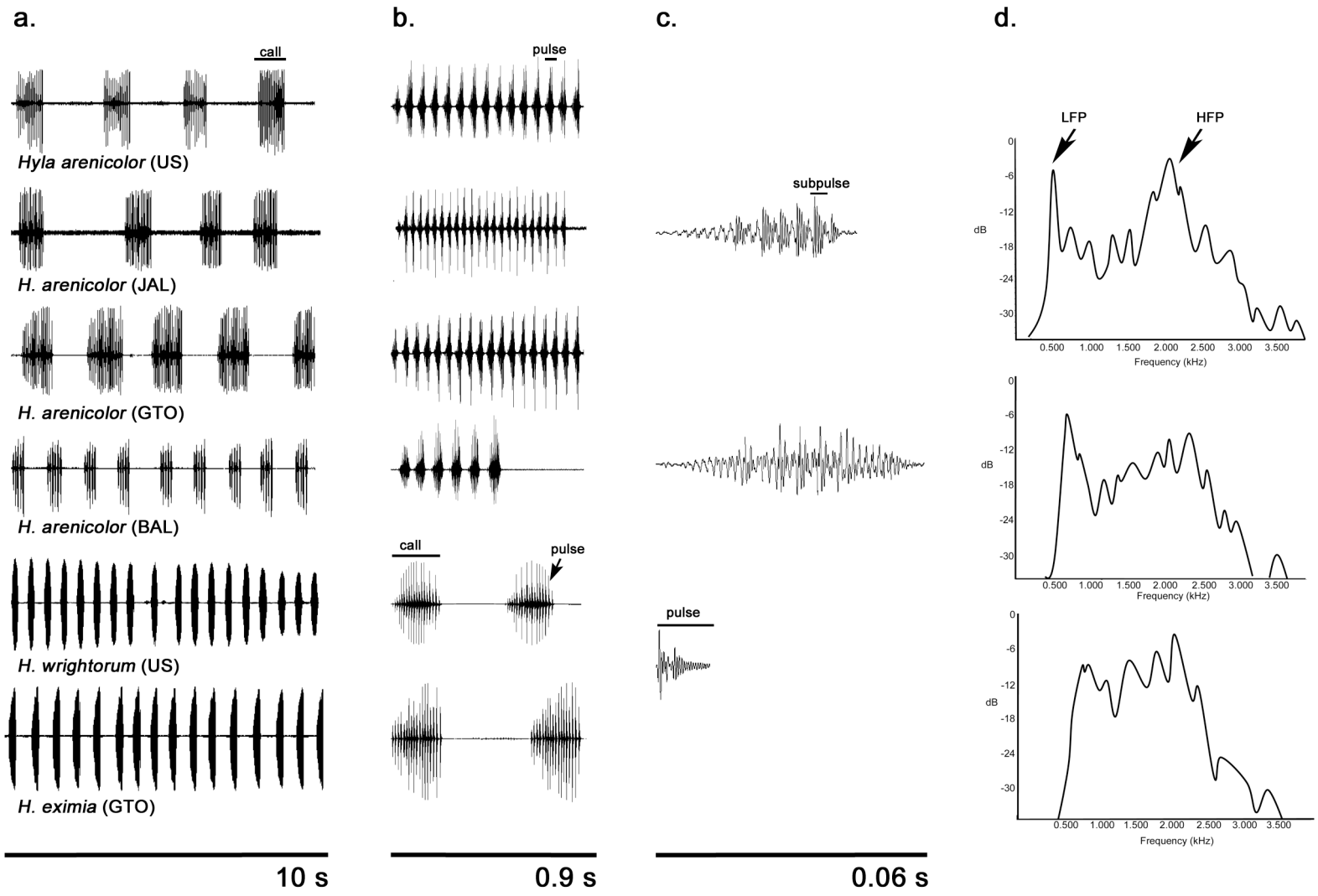

a.
b.
c.
d.
call
pulse
Hyla arenicolor (US)
H. arenicolor (JAL)
H. arenicolor (GTO)
H. arenicolor (BAL)
H. wrightorum (US)
H. eximia (GTO)
subpulse
LFP
HFP
dB
Frequency (kHz)
10 s
0.9 s
0.06 s

Figure 3-2. Oscillograms and power spectra of male advertisement calls.
(a) Oscillograms of ten seconds of calls from *H. arenicolor* USA, CMP-SW, CMP and BAL lineages, as well as calls of *H. wrightorum* (USA) and *H. eximia* (Mexico). (b) A 0.9 second close up of each call revealing the pulsed structure. Notice that *H. wrightorum* and *H. eximia* produce two calls in the same amount of time it takes any *H. arenicolor* to make one call. (c) A close up of an individual pulse showing the sub-pulse structure. The first pulse is representative of the USA, CMP-SW or CMP *H. arenicolor* lineages. The second pulse is from the BAL lineage. The third one is representative of a *H. wrightorum* or *H. eximia* pulse. (d) Power spectra of the calls. Note the bimodal nature of the *H.arenicolor* calls (LFP-Low frequency peak, HFP- high frequency peak). As in (c) the first spectrum is representative of the USA, CMP-SW and CMP *H. arenicolor* lineage, the second is representative of the BAL lineage, and the third spectrum represents *H. wrightorum/H. eximia*.

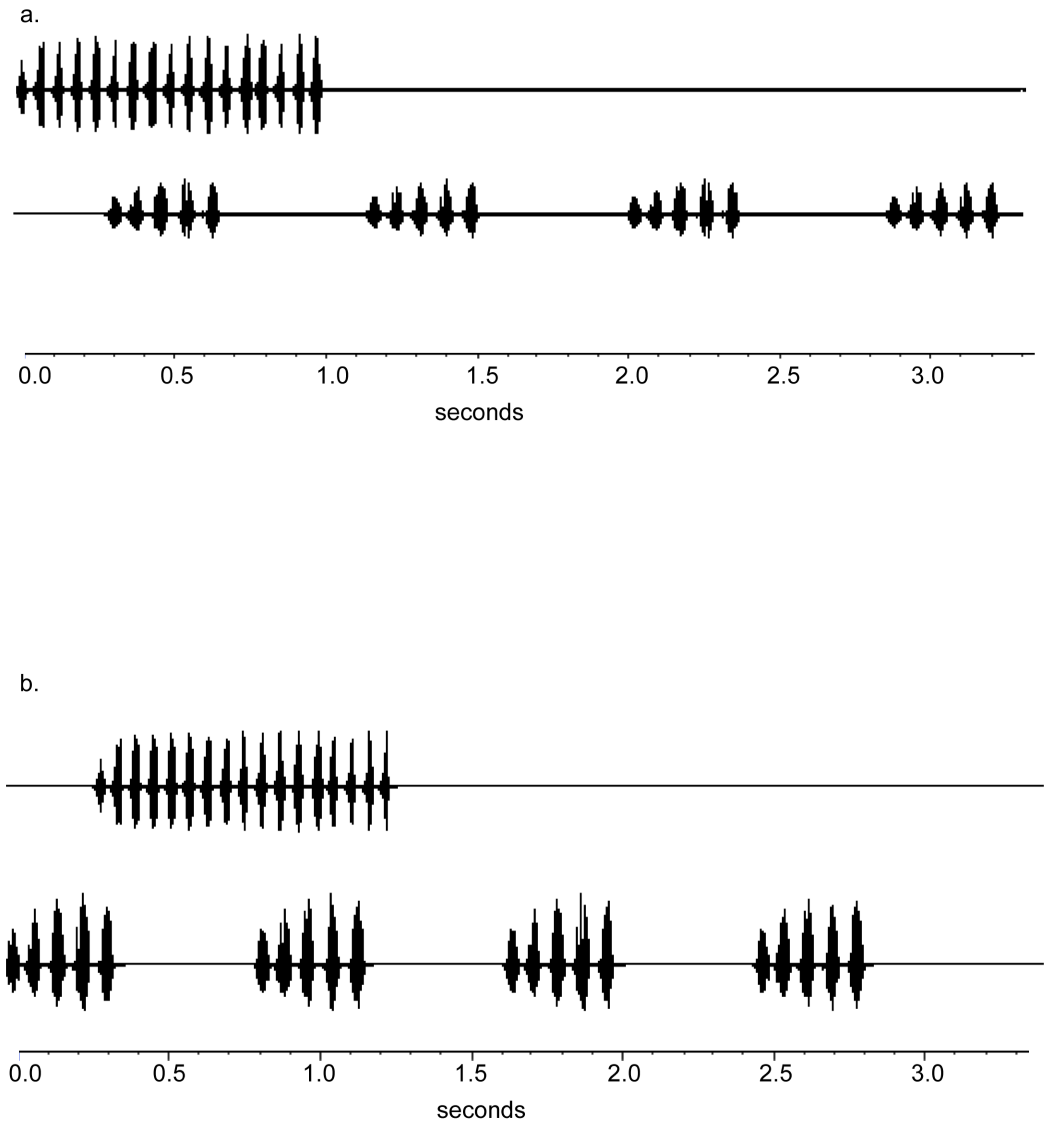


Figure 3-3. Stimuli for the natural CP/GC versus BAL call choice tests.
(a) Top stimulus showing the CP/GC call type leading representing the BAL stimulus.
(b) The top CP/GC stimulus following the bottom BAL stimulus.

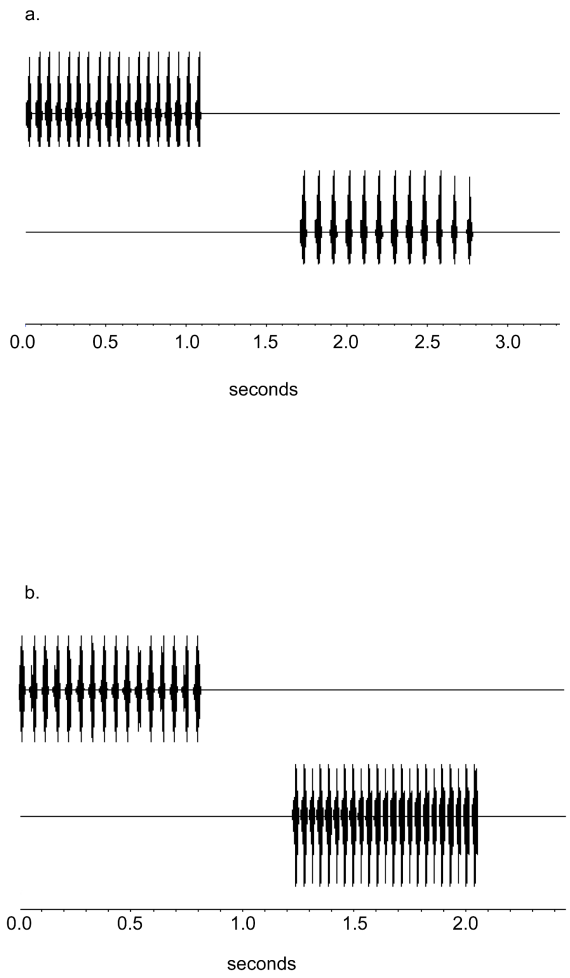


Figure 3-4. Stimuli for pulse rate discrimination tests.
(a) Stimuli for the CP/GC pulse rate (top) versus BAL pulse rate (bottom) choice test.
(b) Stimuli for the CP/GC pulse rate (top) versus CMP-SW pulse rate (bottom) choice test.

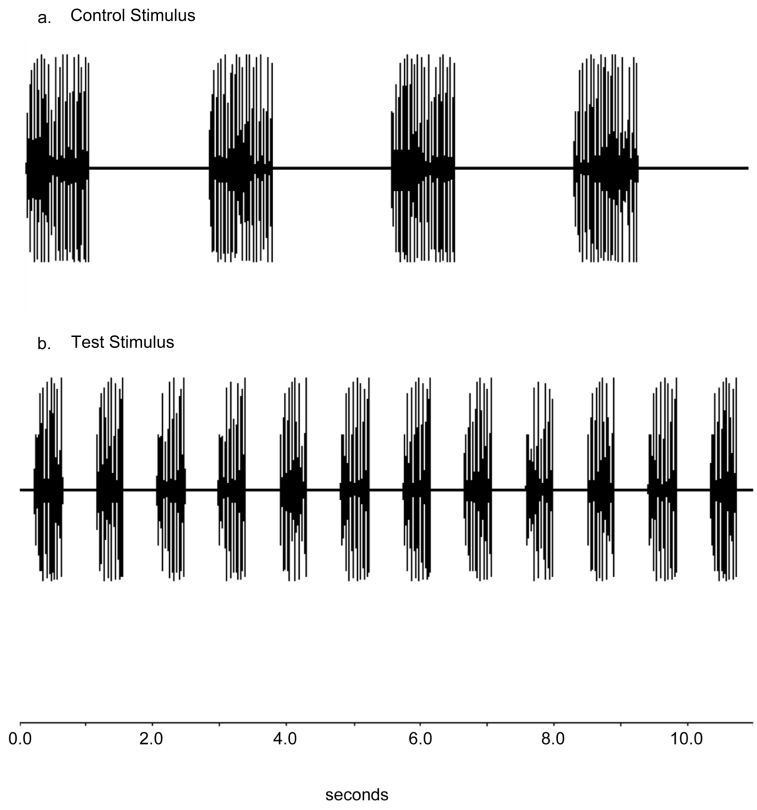
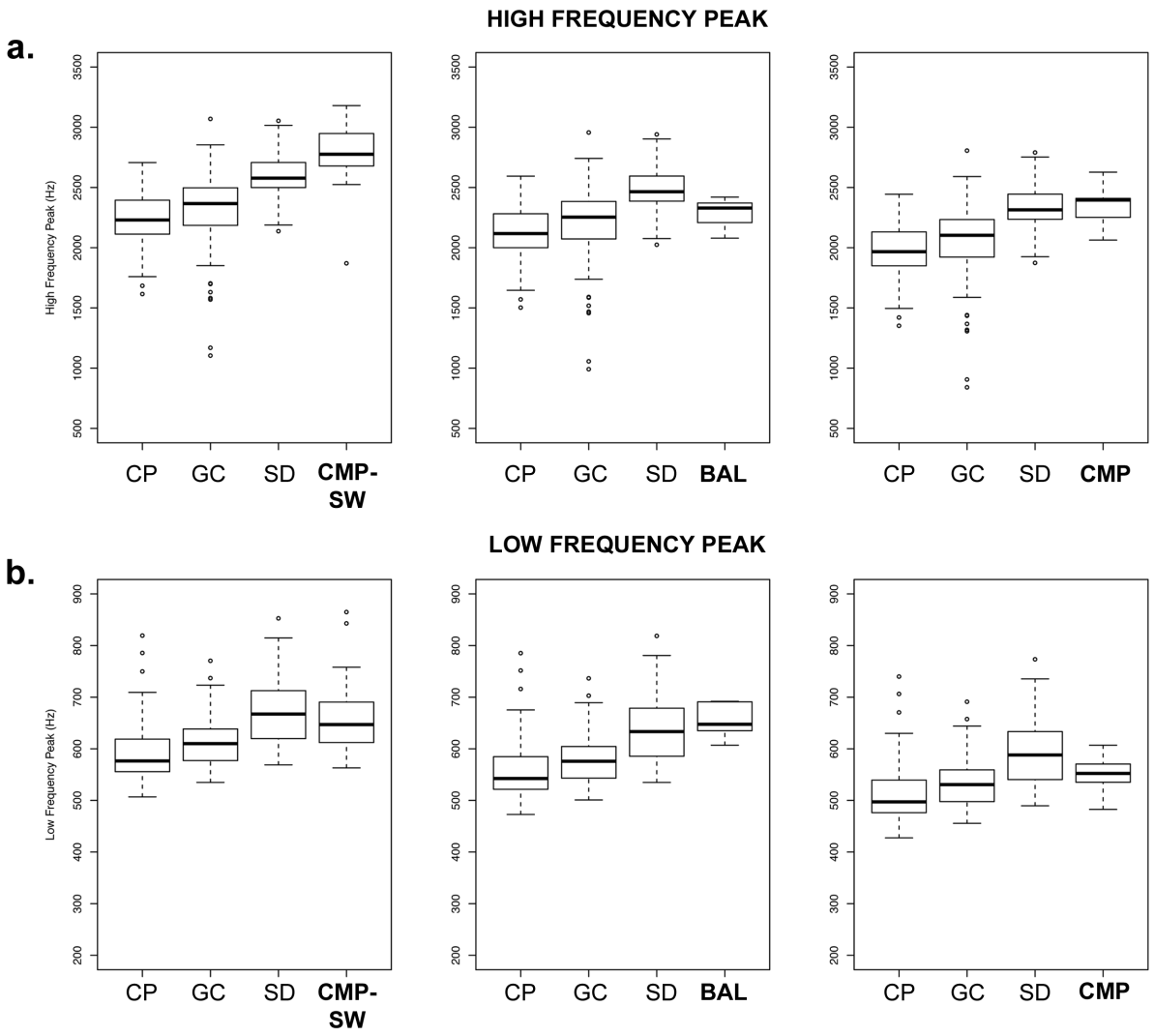
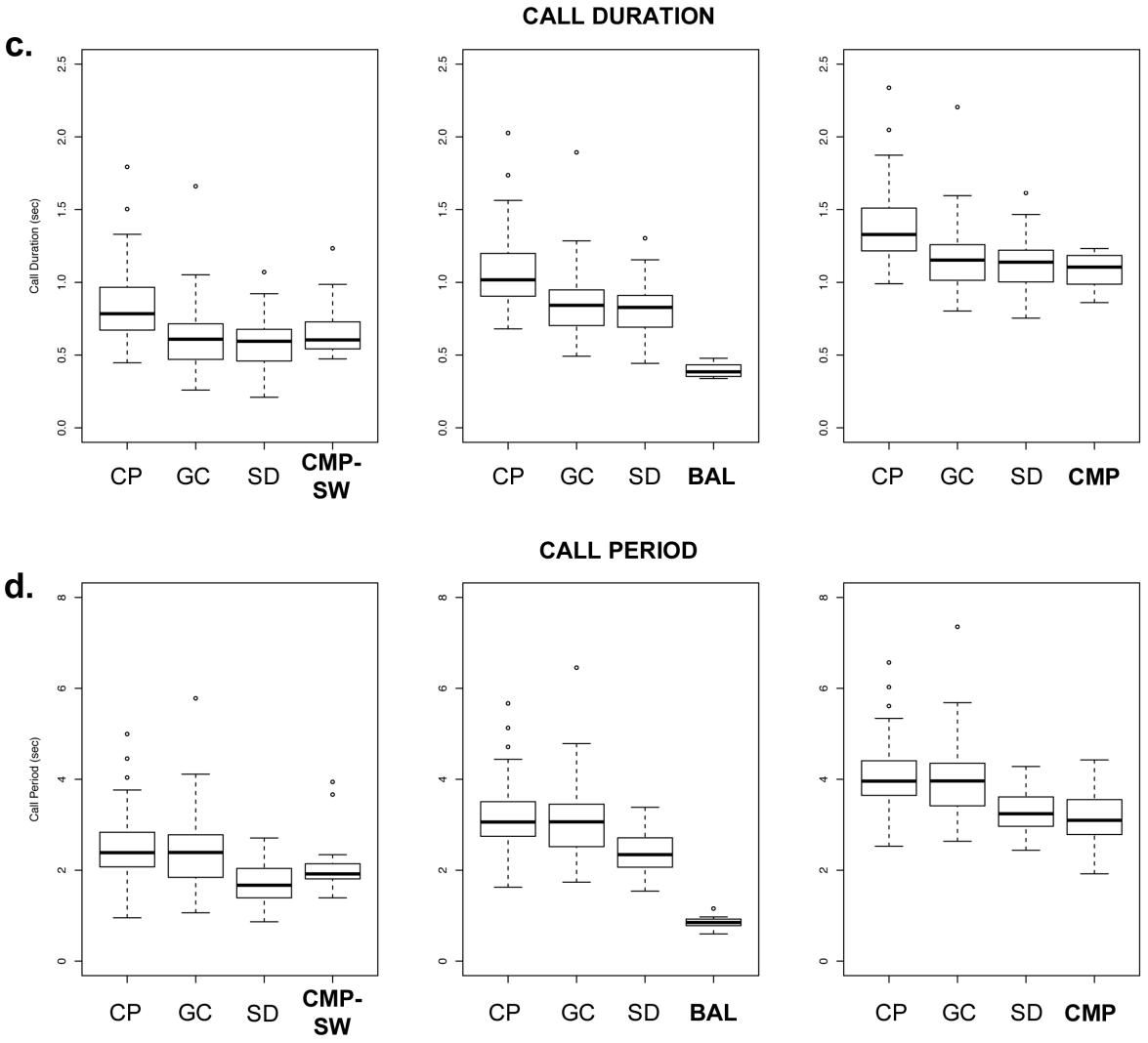
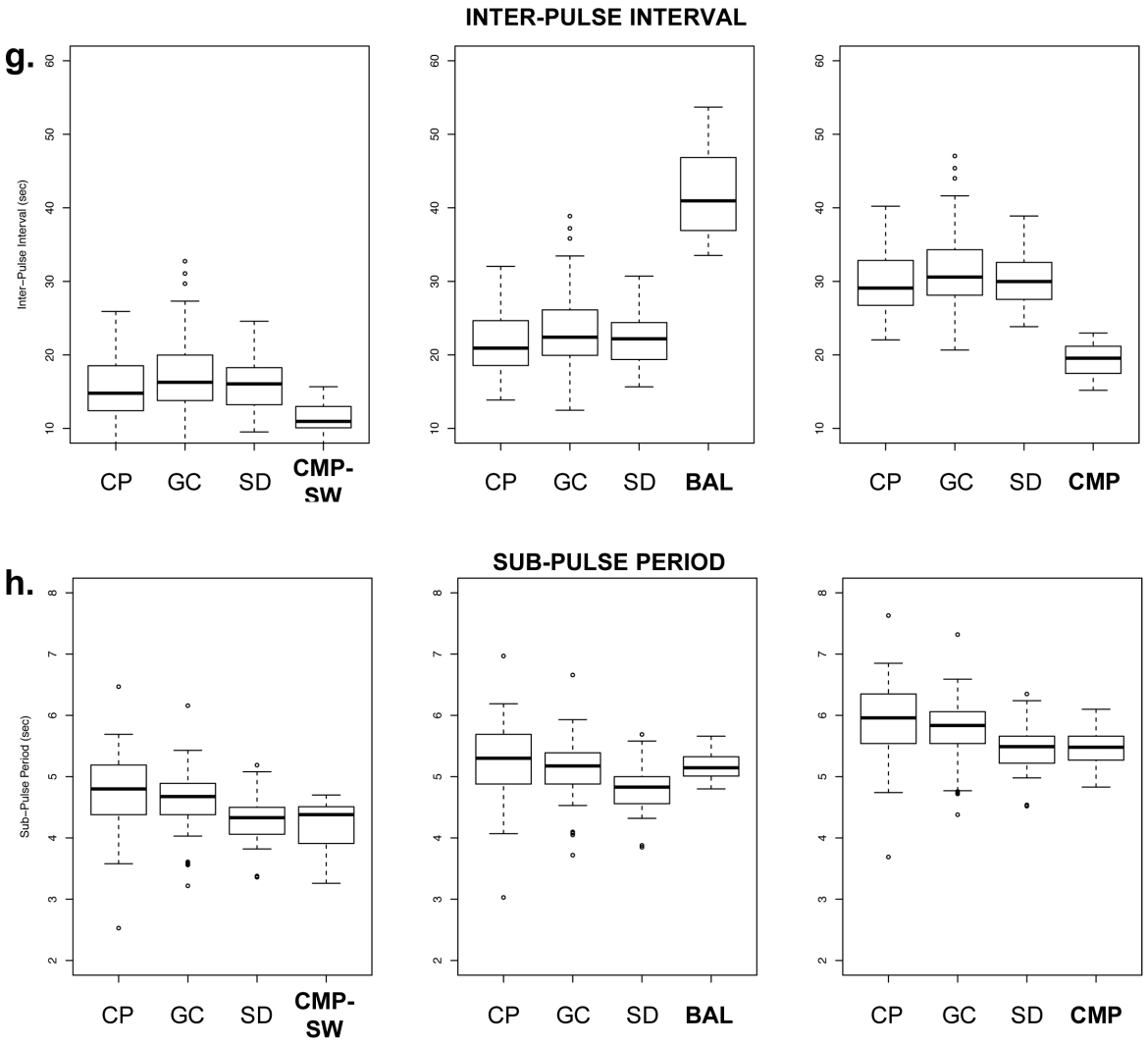


Figure 3-5. Stimuli for the no-choice experiment testing female discrimination against the BAL shorter call duration and shorter call period.
(a) The control stimulus with standard CP/GC call duration and period.
(b) The test stimulus which is the CP/GC call, shortened and repeated at the BAL average values.


a.
HIGH FREQUENCY PEAK
High Frequency Peak (Hz)
500
1000
1500
2000
2500
3000
3500
CP
GC
SD
CMP-SW
BAL
CMP
b.
LOW FREQUENCY PEAK
Low Frequency Peak (Hz)
200
300
400
500
600
700
800
900


c.
CALL DURATION
Call Duration (sec)
0.0
0.5
1.0
1.5
2.0
2.5
CP
GC
SD
CMP-SW
BAL
CMP
d.
CALL PERIOD
Call Period (sec)
0
2
4
6
8


g.
INTER-PULSE INTERVAL
Inter−Pulse Interval (sec)
CP
GC
SD
CMP-SW
BAL
CMP
h.
SUB-PULSE PERIOD
Sub−Pulse Period (sec)

i.

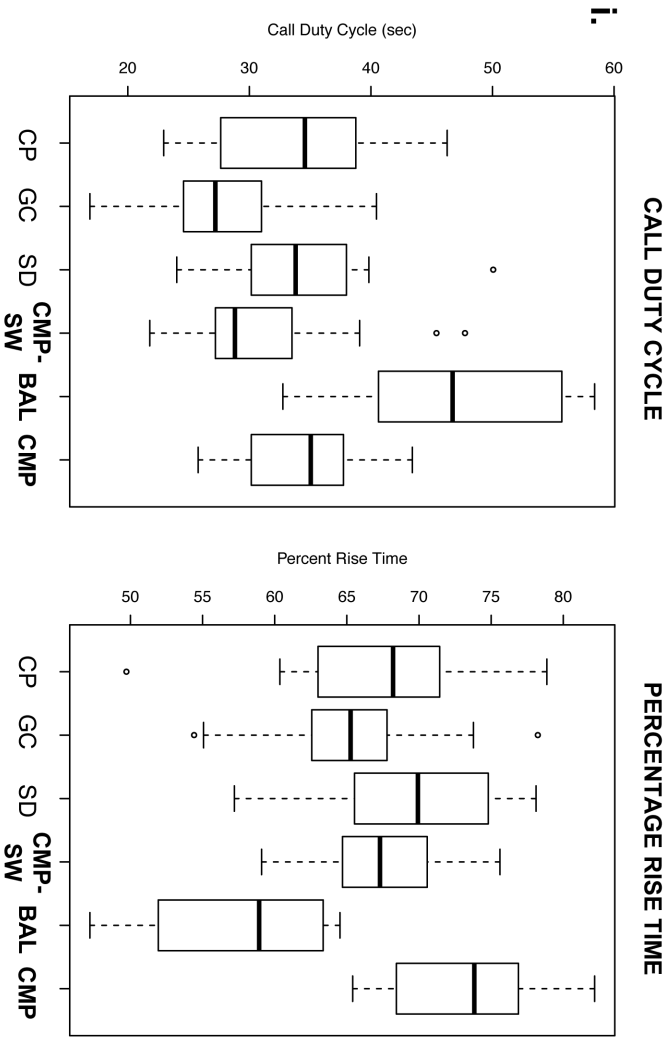

Call Duty Cycle (sec)
20
30
40
50
60
CP
GC
SD
CMP-SW
BAL
CMP
CALL DUTY CYCLE
Percent Rise Time
50
55
60
65
70
75
80
CP
GC
SD
CMP-SW
BAL
CMP
PERCENTAGE RISE TIME

Figure 3-6. Box plots summarizing the median (bar), 25th and 75th percentile (boxes), and 1.5 times the interquartile range (whiskers) for all traits.
Each panel compares a single Mexican lineage with the three USA lineages. Outliers are denoted as circles. (a) low frequency peak (b) high frequency peak (c) call duration (d) call period (e) pulse rate (f) pulse duration (g) inter-pulse interval (h) sub-pulse period (i) call duty cycle and percentage pulse rise-time.

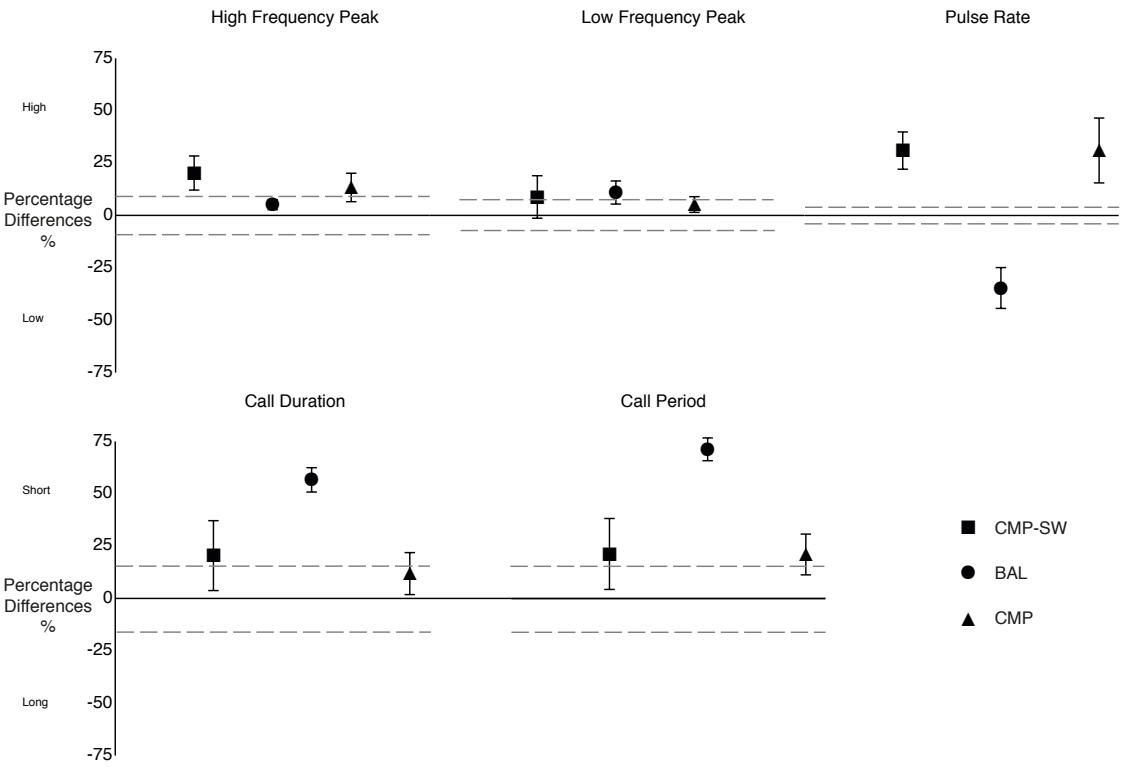


Figure 3-7. Plots showing the average percentage differences and standard deviations in 5 traits (a-high frequency peak, b-low frequency peak, c-pulse rate, d-call duration, and e-call period) among the three Mexican lineages (CMP-SW, BAL and CMP). Percentage differences (y-axis) are relative to the average value for these traits among the three USA lineages (CP, GC, SD). This average USA value is represented as the x-axis on the plot. The dashed gray lines indicate one standard deviation of the average USA value for each trait.

CHAPTER 4

USING AFLP MARKERS TO RESOLVE INTRA-SPECIFIC RELATIONSHIPS AND INFER GENETIC STRUCTURE AMONG LINEAGES OF THE CANYON TREEFROG, *HYLA ARENICOLOR*

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ABSTRACT

The canyon treefrog, *Hyla arenicolor*, is a wide-ranging hylid found from southwestern US into southern Mexico. Recent studies have shown this species to have a complex evolutionary history, with several phylogeographically distinct lineages, a probable cryptic species, and multiple episodes of mitochondrial introgression with the sister group, the *H. eximia* complex. We aimed to use genome wide AFLP markers to better resolve several relationships within this group. As in other studies, our inferred phylogeny not only provides evidence for repeated mitochondrial introgression between *H. arenicolor* lineages and *H. eximia/ H. wrightorum* but also affords more resolution within the main *H. arenicolor* clade than was previously achieved with intron (Bryson et al. 2010) or exon (Klymus et al. 2010) data. However, as with the intron data (Bryson et al. 2010), the placement of a lineage of *H. arenicolor* whose distribution is centered in the Balsas Basin of Mexico remains poorly resolved, perhaps due to past hybridization with the *H. eximia* complex. Furthermore, the AFLP data set shows no differentiation of lineages from the Grand Canyon and Colorado Plateau despite the large mitochondrial differences first reported by Barber (1999a). Finally, our results infer a well-supported sister relationship between this combined Colorado Plateau/ Grand Canyon lineage and the Sonoran Desert lineage, a relationship that again contradicts conclusions drawn from the mtDNA evidence. We discuss several hypotheses to explain these results, and we highlight the importance of multi-taxon (species) sampling in phylogenetic and phylogeographic studies.

INTRODUCTION

Studies of population differentiation can provide insight into the evolutionary processes that drive speciation. Analyzing differences from both phenotypic and genetic perspectives provides clues into the selective, stochastic, and demographic forces that lead to population differentiation. Historical demographic changes (range expansions and contractions) and population connectivity (gene flow and dispersal) can be identified through population genetic studies. The combination of genetic data with current and historical biogeographic information allows one to hypothesize how geographical barriers or conduits to gene flow have affected current ranges (Avise, 2000). Many studies have also examined whether phenotypic change has occurred in conjunction with population differentiation via isolation by distance and drift or whether selective forces have driven phenotypic change (Swallow et al., 2005; Boul et al., 2007; Irwin et al., 2008). However all these studies assume well developed hypotheses of the evolutionary history among populations of the focal species.

Intraspecific phylogeographic and population genetic studies have been dominated by the use of mtDNA because it is rapidly evolving, has a faster coalescence time, and is non-recombinant relative to most known nuclear sequence markers (Avise, 2000; Zink & Barrowclough, 2008). Despite its usefulness in most systems, some studies find incongruent results between the cytoplasmic and nuclear markers. These discrepancies are usually accredited to either to the effect of incomplete lineage sorting or past hybridization and subsequent mitochondrial introgression (Bernatchez, 1995; Glémet, 1998; Sullivan et al., 2004; McGuire et al., 2007; Bossu & Near, 2009; Fontenot, 2011). In fact, recent work has show that within the canyon treefrog, *Hyla arenicolor*,

several episodes of mitochondrial introgression with the sister species complex, *H. eximia* have occurred through the range. Here we define the *H. eximia* complex as being composed of *H. eximia* and *H. wrightorum*, morphologically and behaviorally similar to one another relative to all *H. arenicolor*. Earlier work based solely on mtDNA would have led to erroneous conclusions about phenotypic (male advertisement call) change among some of these lineages (Barber 1999a; Klymus et al., 2010). Despite the confounding effect of mitochondrial introgression in this system, recent studies still reveal strong genetic differentiation of nuclear defined lineages but with unresolved relationships among them (Bryson et al., 2010). Our study's goal is to use genome wide markers (AFLPs) to infer a better resolved intraspecific phylogeny of the canyon treefrog.

A recent study (Bryson et al., 2010) identified several genetically distinct lineages corresponding to geographic regions. Here we use their designations and corresponding abbreviations to refer to *H. arenicolor* lineages: CP-Colorado Plateau (Barber's Clade 1b); GC, Grand Canyon (Barber's Clades 3); SD, Sonoran Desert (Barber's Clade 2); CMP, Central Mexican Plateau (Barber's Clade 1a throughout central Mexico); CMP-SW, Southwestern-Central Mexican Plateau; and BAL, Balsas Basin. Of particular interest is the basal relationship of the BAL lineage of *H. arenicolor* to all other *H. arenicolor* lineages and the lineages that comprise the *H. eximia* complex. This relationship is counter to the expectation, based on call data, that this lineage would be more closely related to the other *H. arenicolor* rather than to the *H. eximia* complex (Klymus et al., unpublished). Furthermore, nuclear introns do not resolve the basal relationships among the main *H. arenicolor* lineages despite strong mitochondrial

differentiation (Bryson et al., 2010). The Grand Canyon lineage shows little nuclear differentiation from the surrounding Colorado Plateau lineage despite having the introgressed mtDNA of the sister species *H. wrightorum* (Bryson et al., 2010; Klymus et al., 2010). We used AFLP markers to better understand phylogenetic relationships among populations and how these relationships influence our understanding of phenotypic differentiation (specifically, male-advertisement call) in this system.

METHODS

Taxon Sampling

We collected tissue samples of *H. arenicolor*, *H. wrightorum* and *H. eximia* in the field from 2004 – 2009 and obtained additional samples from museum collections as indicated in Table 4-1. Sampling localities are indicated on Fig. 4-1, in which the mitochondrial lineages for each sample correspond with those of Bryson et al. (2010). The outgroup species *Hyla chyrsoscelis* and *Hyla femoralis* were used as they are in a clade sister to the ingroup based on molecular evidence (Faivovich et al., 2005; Wiens et al., 2010).

Molecular Markers

Total genomic DNA was extracted from EtOH-preserved toe or liver tissue with the DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA) following standard procedures.

AFLP

Amplified fragment length polymorphism profiles were generated using a modified version of the protocols of Vos et al. (1995). A detailed protocol and example electropherograms are provided in Appendices D and E. In one digestion/ ligation step,

whole genomic DNA was digested with the restriction enzymes EcoRI and MseI (New England Biolabs, Ipswich, MA) and adaptors were ligated to the overhanging ends of the digested product using T4 DNA ligase (New England Biolabs, Ipswich, MA). Diluted product was used in a pre-selective amplification reaction using two pre-selective primer combinations: E_A/M_A or E_A/M_C. Final selective amplifications were performed using six primer pair combinations with the Eco primer being fluorescently labeled: E_{ACT}/M_{ATA}, E_{ACT}/M_{ACA}, E_{ACT}/M_{CAC}, E_{ACT}/M_{CAT}, E_{ACT}/M_{CAA}, E_{AAC}/M_{CAA}. Two additional combinations were used when testing primer combinations (E_{AAC}/M_{ACA}, E_{AAC}/M_{ATA}) but only the above six pairs gave consistent results and were used in subsequent analyses. Each primer pair was amplified in separate selective amplification PCR reactions. Amplification products were genotyped at the DNA Core Facility, University of Missouri, on an ABI 3730 genetic analyzer with a Liz 600 internal size standard (Applied Biosystems, Foster City, CA, USA).

Genotypes were analyzed in GENEMARKER v 1.95 (Softgenetics LLC, State College, PA, USA). Fragments were scored using the AFLP analysis tool in GENEMARKER, and were manually checked by a single observer (K. Klymus). Only fragments between 70 to 600 base pairs were scored to avoid excessive homoplasy of small fragments (Vekemans et al., 2002), and only fragment peaks with intensities greater than 100 were scored. Results were exported as a binary matrix of fragment presence or absence.

To assess methodological error rate, 12 individuals (10% of the total sample size) were run twice through the selective amplification step. Allelic error rates were calculated according to Bonin et al. (2004) as the total number of differences observed

between repeats over the total number of alleles. As suggested by Bonin et al. (2007), we removed fragments that had a per-locus error rate greater than 0.1%. This resulted in removing 90 of the total 817 fragments, giving a final data matrix of 727 fragments (Appendix F). The overall allelic error rate of the final data set was 2.21%, which is within the range of other studies (Bonin et al., 2004).

mtDNA

An approximately 2.5 kb segment of mitochondrial 12S and 16S genes and the intervening valine tRNA were amplified with a set of overlapping primers (Appendix A) for a subset of individuals (Table 2, Goebel et al., 1999; Pauly et al., 2004).

PCR amplifications of the mitochondrial fragments were performed with the following thermocycler protocol: 2 min at 94° C, denaturing for 30 sec at 94° C, annealing for 30 sec at 47° C, extension for 1 min at 72° C, and final extension for 7 min at 72° C; denaturing, annealing and the first extension stage were cycled 35 times. Products were visualized with a 2% agarose gel and were purified using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Sequencing reactions were completed with an automated DNA sequencer (ABI 3730 and 3700 analyzers).

Mitochondrial fragments were combined to give a contig of 2,488 bp. Alignment was performed using Clustal X (Larkin et al., 2007). Very few regions were difficult to align, and these were adjusted manually to minimize the inferred number of evolutionary changes.

Phylogenetic Analysis

AFLP

The AFLP character matrix was analyzed using distance, maximum parsimony and Bayesian optimality criteria. For the distance analysis, the data were transformed into a Nei-Li genetic distance matrix (Nei & Li, 1979), which was subsequently used in a clustering algorithm to estimate a neighbor-joining tree in PAUP* 4.0b10 (Swofford, 2002) with 2000 bootstrap replicates for support. Distance methods provide overall phenetic relationships based on similarity, whereas character methods allow for the inference of phylogenetic relationships by assuming derived character states. Parsimony dictates that the best tree is the one that requires the fewest character state changes (Felsenstein, 1983). Parsimony analysis was performed in PAUP* 4.0b10 (Swofford, 2002) using Wagner maximum parsimony criteria and a heuristic search with 100 random addition replicates and TBR branch swapping. Nodal support values were obtained through bootstrapping with 2000 pseudoreplicates.

Bayesian approaches allow for the acceptance of non-parsimonious trees, and can incorporate more intricate evolutionary models in the tree searching process (Koopman et al., 2008). However, AFLP data sets include only loci for which at least one fragment is present in a genotype. This creates a bias in the data set since it is also possible for the entire locus to be absent in an individual, not just an allele (Koopman et al., 2008; Snyder et al., 2009). Thus, modeling evolutionary change in AFLP data sets has proved difficult. However, some studies use a F-81 like model to analyze such data (Albach, 2007), and the addition of the 'noabsencesite' option to the model accounts for the inherent coding bias (Koopman et al., 2008; Snyder et al., 2009). Although a more intricate model of

AFLP evolution has been created (Luo et al., 2007), it is computationally too burdensome for most analyses (Koopman et al., 2008).

We conducted Bayesian analyses in MRBAYES 3.1 (Ronquist & Huelsenbeck, 2003) using the F-81 model with the 'noabsencesite' option. Bayesian settings included two runs with 4 chains each (3 heated and one cold), sampling every 1000 generations for 60 million generations. A burnin of 15,000 trees was used before calculating posterior probabilities. We experimented with different temperature settings (0.05-0.25) for the heated chains and found that the default setting of 0.20 provided adequate mixing of the chains and convergence. Convergence of likelihood scores was assessed by plotting the log-likelihood values of the cold chain as well as looking for a split frequency of less than 0.01.

mtDNA

Phylogenetic analyses of the mtDNA data set were performed using maximum parsimony and Bayesian methods. Maximum parsimony trees were generated using PAUP* 4.0b10 (Swofford, 2002). The mtDNA data set was analyzed using heuristic searches with 100 random addition sequences and TBR branch swapping. Bootstrap values were calculated based on 2000 pseudoreplicates.

For the Bayesian analysis, MRMODELTEST 2.3 (Nylander, 2004) was used to select a best-fit model of nucleotide substitution. The selected model according to the Akaike Information Criterion (AIC) was: GTR+G+I. Bayesian analysis was implemented in MRBAYES 3.1 (Ronquist & Huelsenbeck, 2003). The MCMC settings included two runs of four chains each (three heated and one cold chain), sampling every 1000 generations for ten million generations. Posterior probabilities were calculated after a

burnin of 2500 trees for each of the four runs and convergence was assessed in the same manner as it was for the AFLP data set. Initial analyses revealed that the estimated branch lengths from our Bayesian analysis were longer than those estimated by maximum likelihood analyses (not shown) and were biologically unreasonable. Other studies have reported a bias towards long-branch estimates in Bayesian models (Brown et al., 2010; Marshall, 2010), and researchers have begun testing the affects of branch length priors on their dataset (McGuire et al., 2007; Bryson et al., 2010). We found that a branch length prior of 50 resulted in a tree with branch lengths similar to those observed on ML trees. Changing branch length priors did not affect the tree topology.

Population Genetic Analysis-AFLP

Genetic differentiation statistics were calculated using the AFLP data matrix under the assumptions that co-migrating fragments are homologous loci and that populations are at Hardy-Weinberg genotypic proportions. Allelic frequencies were estimated using Zhivotovsky's (1999) Bayesian method with uniform prior distribution as implemented in the program AFLP-SURV v 1.0 (Vekemans 2002). The program then calculates estimates of genetic diversity (H_e) and between-population unbiased estimates of Nei's (1972) using the approach of Lynch and Milligan (1994). The data matrix for these analyses excluded individuals from the two outgroups and the one CMP individual.

To examine patterns of population structure we used the Bayesian clustering methods implemented in the program STRUCTURE v 2.3 (Pritchard et al., 2000). The latest version of STRUCTURE allows for the use of the admixture model with correlated allele frequencies to assess the number of clusters (K) with dominant markers (Bonin et al., 2007; Falush et al.,2007). We ran STRUCTURE for 100,000 iterations after a burnin of

10,000 iterations, estimating K from one through ten, each with ten runs. The data matrix used in STRUCTURE included three artificially crossed hybrids (*H.arenicolor*-SD lineage and *H. wrightorum*) as well as the one CMP individual.

RESULTS

Phylogenetic Analyses

Our AFLP analyses included 727 alleles of which 709 (98%) were polymorphic and parsimony informative. All analyses based on the AFLP data (distance, MP and Bayes) yielded similar topologies (Fig. 4-2 and Fig. 4-3). Our mtDNA phylogeny (Fig. 4-3) gave results similar to those of other studies (Bryson et al., 2010; Klymus et al., 2010) in that two *H. arenicolor* lineages (GC and CMP-SW) clustered with the *H. wrightorum* or *H. eximia* lineages, respectively. Pairwise p-distance values from mtDNA sequence data are provided in Table 4-3.

AFLP NJ tree

The neighbor-joining tree placed all ingroup individuals in a well-supported clade with a bootstrap value of 96 (Fig. 4-2). Furthermore, we found support for the monophyly of *H. arenicolor* including the BAL lineage. Our AFLP data provide further resolution among the *H. arenicolor* lineages. As expected from previous nuclear work (Bryson et al., 2010; Klymus et al., 2010), the CP and GC lineages are grouped together with strong bootstrap support, and there is very little evidence of differentiation within this CP/GC lineage. Within the main *H. arenicolor* clade, there is a highly supported sister relationship between SD and the CP/GC lineages. The CD and CMP-SW lineages, though well supported themselves, are in a polytomy with the CP/GC/SD group.

AFLP MP/ Bayes tree

Phylogenetic relationships inferred from both maximum parsimony and Bayes analyses have similar topologies. Figure 4-3 shows the AFLP Bayesian tree with both Bayesian posterior probability and maximum parsimony bootstrap values in parentheses at each node. Overall, the topology is similar to that of the phenetic neighbor-joining tree; however, some nodes show lower support. As before, all *H. arenicolor* including the BAL lineage are grouped into one clade; however, the support values are not high, with a posterior probability of 0.82 and a bootstrap value of 72. The main *H. arenicolor* lineages, however, are strongly supported as a monophyletic lineage sister to the BAL lineage. As in the neighbor-joining tree, the CP and GC lineages are indistinguishable and fall into one well supported clade Furthermore, as in the phenetic analysis, this group is strongly supported as sister to the SD lineage. Outside of *H. arenicolor*, *H. wrightorum* and *H. eximia* are both well supported lineages; however, support for their sister placement with one another is low, with a posterior probability of 0.72 and no support (polytomy) for the MP bootstrap analysis. Despite the lack of resolution among these main groups (the main *H. arenicolor*, the BAL lineage, *H. wrightorum* and *H. eximia*) each are well supported as distinct lineages.

mtDNA MP/ Bayes tree

Both maximum parsimony and Bayesian analyses of the mtDNA data produced the same, well-supported topology that is consistent with previous mtDNA studies (Bryson et al., 2010; Klymus et al., 2010). As in previous studies, our mtDNA results are incongruous with the relationships found in our nuclear (AFLP) data set. Our mtDNA tree supports the results of Bryson et al.'s (2010) study using ND4, ATPase 6, ATPase 8,

and flanking tRNA mtDNA sequence. As in Bryson et al.'s (2010) study we have a CP group that is sister to the CD and CMP lineages (note we only have one CMP individual in our study). This group is sister to the SD lineage. Outside this group the *H. arenicolor* GC lineage is embedded within *H. wrightorum*, and the *H. arenicolor* CMP-SW lineage is sister to *H. eximia*. Furthermore, the BAL lineage groups with this *H. eximia*/ *H. wrightorum* group. Additionally, two *H. arenicolor* individuals (00017H and 00049H) found in the CP/GC group according to the AFLP phylogeny are placed within the CD mtDNA lineage (Fig. 4-3).

AFLP population genetic statistics

Results from AFLP-SURV found that the number of polymorphic loci per lineage ranged from 14.9 % to 49.5%. The CP and GC lineages had the lowest percentage of polymorphic loci (18.8% and 14.9 % respectively) as well as the lowest heterozygosity (He) (Table 4-4). Very few private alleles were found among populations (Table 4-4); however, the BAL lineage had many more than any other lineage, with 20 private alleles. Pairwise Fst and Nei's genetic distance (Lynch & Milligan, 1994) values between populations (Table 4-5) reveal very high pairwise genetic distances except between the CP and GC lineages.

In interpreting results from STRUCTURE, we examined both the LnP(D) and Δ K as suggested by Evanno et al. (2005). Both of these values were highest for a K=7 (Table 4-6) . These seven clusters correspond to CP/GC, SD, CD, CMP-SW, BAL, *H. wrightorum*, and *H. eximia*, consistent with results of the AFLP phylogenetic analyses. Plots of the STRUCTURE results show each cluster to be very homogenous with little mixing of alleles from other clusters (Fig. 4-4). Our three hybrids showed equal portions

of the *H. arenicolor* SD lineage and *H. wrightorum*, as would be expected for an F1 hybrid. Furthermore, the one CMP individual included in the analysis grouped with the CD lineage, but shared alleles with CMP-SW and some with *H. eximia*. Finally, results for some individuals showed a consistent sharing of alleles with other lineages for all ten runs (Fig. 4-4). We also analyzed the CP and GC lineages for fine-scale divergence between these two mtDNA distinct lineages. We found no structure, with a K=1 having the highest LnP(D).

DISCUSSION

Balsas Lineage

Our original goal was to resolve the relationships among the main *H. arenicolor* lineages and the BAL lineage. Although originally described as a population of canyon treefrogs (Pierce, 1968), intron sequence data later placed the BAL lineage sister to a combined group of *H. arenicolor*, *H. wrightorum* and *H. eximia* (Bayesian posterior probability = 0.82) (Bryson et al., 2010) (Fig. 4-5). These results make *H. arenicolor* paraphyletic, and suggest that this group exhibits an extreme level of morphological and behavioral conservation or convergence conservation between the main *H. arenicolor* and the BAL lineages. Though biologically significant male advertisement call differences exist between the BAL lineage and all other *H. arenicolor*, the calls of the BAL lineage are still qualitatively similar to other *H. arenicolor* (Pierce, 1968; Klymus et al., unpublished) and are distinctly unlike those of the *H. eximia* complex (Gergus et al., 2004; Klymus et al., unpublished). Finally, similar morphology groups the BAL lineage with all other *H. arenicolor* rather than with the *H. eximia* complex (Pierce, 1968;

Duellman, 1970). All *H. arenicolor*, including the BAL lineage, have a plump appearance with a mostly gray-tan colored, tuberculate skin. In comparison, the *H. eximia* complex frogs are green or brown in coloration and have a more slender body with smooth skin.

We hypothesized that the Bayesian posterior probability support for BAL lineage's placement in Bryson et al.'s (2010) study was not strong enough to support this conclusion. In general, Bayesian posterior probabilities that are 0.95 or higher are considered to represent a high level of clade support (Erixon et al., 2003; Shaffer et al., 2004; Simmons et al., 2004). We believed more nuclear markers would resolve the BAL lineage's puzzling placement. Contrary to previous results, our AFLP markers group all *H.arenicolor*, including the BAL lineage, in a monophyletic clade (Figs. 4-2, 4-3, and 4-5) as supported by behavior and morphology. Yet once again, this placement is not strongly supported with a Bayesian posterior probability of 0.82 and bootstrap support of 72.

We propose two possible interpretations of our data. First, the lack of strong support with either data set might suggest that these relationships are unresolvable by our AFLP markers and intron sequences. However, mtDNA data infer well-supported deep divergences among these lineages, it seems unlikely that neither intron nor AFLP markers could provide some resolution to such deep divergences. We believe a more likely alternative is that hybridization among these lineages has obscured phylogenetic signal. In fact one of our *H. eximia* samples shares alleles with the BAL lineage (Fig. 4-4), suggesting past gene flow. Based on behavioral and morphological evidence, we therefore suggest that the BAL lineage is likely to be more closely related to other *H.*

arenicolor than to the *H. eximia* complex. Nevertheless, the BAL lineage exhibits the largest Fst values, indicating genetic isolation and strong differentiation from other lineages. These data lend support to previous genetic (Bryson et al., 2010) and behavioral (Pierce, 1968; Klymus et al., unpublished) evidence that the BAL lineages is a distinct species

We propose that this lineage became isolated from other *H.arenicolor* by a biogeographical barrier. The BAL lineage is found in the Balsas Basin and surrounding mountains, an area known for its high level of endemic taxa (Becerra & Venable, 1999; Zaldívar-Riverón et al., 2004; Lott & Atkinson, 2006). This dry lowland depression likely formed from uplifting of the trans-Mexican volcanic belt and the Sierra Madre del Sur mountain ranges (Devitt, 2006). Including some of the highest mountains in Mexico, the Trans-Mexican Volcanic belt and resulting Balsas Basin act as a biogeographic barrier to many taxa (Marshall & Liebherr, 2000). Uplift of the trans-Mexican volcanic belt was thought to begin in the late Oligocene and continuing throughout the Miocene and into the Pliocene and Pleistocene, with later episodes of orogeny believed to close off the basin (Halffter, 1987; Becerra, 2005; Bryson et al. 2011). Divergence date estimates for the Balsas lineage are concordant with Miocene uplifting. Although no other *H. arenicolor* have been found in the Balsas Basin, Bryson et al. (2010) sampled individuals from the BAL and CMP-SW lineages within less than 100 km of one another in the surrounding mountains. This suggests that these lineages may not be currently isolated by geography; however, behavioral isolation due to female preference for advertisement call differences may reduce the likelihood of gene flow (Klymus et al., unpublished).

Increased sampling from the Trans-Mexican volcanic belt and Balsas Basin is needed to determine more precisely potential contact zones.

CP/ GC Lineages

Of further interest to our study was the delineation between the CP and GC clades. Nuclear sequence data suggest that they comprise one homogeneous lineage, contrary to the conclusion based on mtDNA data. Since the introgressed mitochondrial genome of *H. wrightorum* found in the GC lineage has not been observed in adjacent populations, gene flow is apparently no longer occurring between these populations. We used AFLP markers to search for fine-scale differentiation that may indicate recent isolation. Contrary to our expectations, however, the AFLP data do not support independent lineages within this group (Fig. 4-2, 4-3, and 4-4). Furthermore, AFLP-inferred Fst and genetic distance values between the CP and GC lineages are low and hence indicative of little differentiation and recent gene flow (Table 4-5) (Fig. 4-4).

Two hypotheses may explain our results. First, female philopatry and male-biased dispersal can lead to patterns of geographically distinct mitochondrial lineages persisting even with nuclear gene flow (Prugnolle & de Meeus, 2002; Johansson et al., 2008,); however, limited evidence for male-biased dispersal in anurans exists (Lampert et al., 2003). Comparison of Fst values or assignment tests between the two sexes may also allow us to determine whether sex-biased dispersal has led to the observed pattern (Prugnolle & de Meeus, 2002). Unfortunately, most of our sampled individuals were not identified to sex, so we could not test this hypothesis. Second, this pattern may exist if divergence between the CP and GC lineage was too recent to be resolved by our AFLP markers. Because data suggest no differentiation between the CP and GC lineages, we

can assume that isolation is very recent and likely to have occurred close to the time of the introgressive event between the GC lineage and *H. wrightorum.* Average pairwise uncorrected p-distances between the GC lineage's mitochondrial sequence and *H. wrightorum's* mitochondrial sequence are low suggesting a recent event (Table 4-3). We can thus infer a relatively recent divergence between the CP and GC lineages. If so, nuclear markers may not be able to resolve any genetic divergence. Comparison with a similar pattern in the CMP-SW and adjoining CMP lineages in Mexico could be useful for testing this possibility. An ancient hybridization event involving the introgression of *H. eximia*'s mitochondrial genome into the CMP-SW lineage was detected by Bryson et al. (2010) and is also supported by our data (Fig. 4-3). Sampling from the CMP lineage may enable us to determine the level of nuclear gene flow between these seemingly isolated mitochondrial lineages. Because this introgressive event is much older than that in the Grand Canyon, we expect that molecular markers would resolve a divergence between the introgressed lineage (CMP-SW) and the adjacent (CMP) lineage. Differentiation between these lineages by nuclear markers would support a hypotheses that the introgressive event in the GC is simply too recent for the markers to resolve. If, however, high levels of gene flow are present between the CMP-SW and CMP, this would support the male-biased dispersal hypothesis. We could not test these hypotheses, as we only had one CMP individual. However our STRUCTURE results for the one CMP individual does indicate mixed ancestry (or allele sharing) with the CMP-SW lineage and CD lineages, suggestive of potential gene flow in the CMP lineages that was undetected by analyses of mtDNA sequence (Fig. 4-4). Further sampling of the CMP lineage is needed, and faster evolving nuclear markers would also allow us to more definitively

distinguish between these hypotheses in both the CP/ GC and the CMP-SW/CMP scenarios.

SD Lineage

Finally, our study resolves relationships within the main *H.arenicolor* clade that are incongruous with expectations derived from the mtDNA data. Specifically, mtDNA analysis indicates that the CP/GC and CD lineages are sister to one another. Our mitochondrial divergence estimates are consistent with other studies using various mtDNA markers with this species (Murray, 1997; Barber, 1999a; Bryson et al., 2010). All previous studies infer deep sequence divergence among the lineages, including the SD lineage. Nuclear intron data place all main clade *H.arenicolor* lineages in a polytomy (Bryson et al., 2010) (Fig. 4-5), suggesting that intron sequences cannot resolve relationships among the lineages. Interestingly, our AFLP data set strongly supports a sister relationship between the CP/GC and SD lineages rather than between the CP/GC and CD lineage as might be expected based on the mtDNA gene tree (Figs. 4-2, 4-3 and 4-5).

We present three possible explanations for the apparent incongruent nature of our genetic markers. First this pattern could be a result of incomplete lineage sorting among the nuclear AFLP markers or that the levels of mtDNA divergence are representative of current diversity. Nuclear loci have a larger effective population size (N_e) and subsequently take a longer time to coalesce relative to mtDNA loci (Moore, 1995). This would suggest that our AFLP data cannot accurately resolve the placement of *H. arenicolor* lineages. In a similar fashion, these high levels of mtDNA divergence may simply reflect within species haplotype diversity. Studies on mammals have found highly

divergent mtDNA haplotypes (cytochrome-b 8%) within not just the same species, but the same population (Wayne et al., 1990). This level of within species mtDNA variation, however, appears to be unusual with regard to anurans. Anuran intraspecific cytochrome-b divergence is generally < 3% (Graybeal, 1983; Holloway et al., 2006). In comparison, cytochrome-b sequence divergence between the SD (Clade 2) and CP (Clade 1) lineages in Barber's (1999a) study was 9.2%. Other exceptions to this general trend include lineages of red-spotted toads (*Bufo punctatus*) (Jaeger et al., 2005) and tailed frogs (*Ascaphus truei*) (Nielson et al., 2001), but further investigation with nuclear markers is needed to determine if these lineages also exhibit corresponding nuclear differentiation. We feel these hypotheses do not explain our data because the large mtDNA divergences among lineages suggests that there has been adequate time for lineage sorting, and our AFLP data provide strongly supported relationships among lineages but they are inconsistent with what we would expect based on the mtDNA phylogeny.

Because we are confident that our mtDNA data are robust, perhaps homoplasious fragments are leading to an inaccurate phylogenetic signal in our AFLP data set. Homoplasy of AFLP markers likely increases with increasing genetic divergence (Althoff et al., 2007); thus it is possible that even the main *H.arenicolor* lineages are too divergent for proper resolution with AFLP markers. Recent studies, however, have used AFLPs to resolve divergences as deep as 15 mya in pinnipeds (Dasmahapatra et al., 2009) or, in the case of snubnose darters, cytochrome-b sequence divergences of 17.4% (Mendelson & Wong, 2010). These studies suggest that the relatively shallower divergences observed in our study system should be resolvable by AFLPs.

Confident in the phylogenetic signal of both our genetic markers, we suggest an alternative explanation, that the SD lineage has the introgressed mitochondrial genome of an unsampled, extinct lineage. Holloway et al. (2006) suggested that hybridization between extant and extinct lineages of gray treefrogs explain the high amounts of intraspecific sequence divergence observed in their study (2 -3.5 % cytochrome-b). The much higher levels of mitochondrial divergence between the CP and SD lineages of canyon treefrogs (9% cytochrome-b; Barber, 1999a) are well above differences observed within other North American hylids (Holloway et al., 2006), supporting this scenario. This hypothetical lineage would have been highly divergent from the other *H. arenicolor* lineages but would fall within a monophyletic *H.arenicolor* complex sister to the *H. eximia* group (Fig. 4-6). More specifically, a CP/GC/SD lineage would have hypothetically split from the CD/CMP and CMP-SW lineages. The CP/GC and SD lineage would have then diverged, and the SD lineage would have come into contact with this highly divergent lineage, followed by mtDNA introgression (Fig. 4-6). This scenario would have resulted in the loss of the SD's original mitochondrial genome, which would have then been replaced by the extinct lineage's mitochondrial genome. A northward expansion of the CD lineage would have then resulted in close proximity to both the CP/GC and SD lineages, as is seen today. Support for such an expansion of the CD lineage north of the Chiricahuas was inferred by Barber (1999b).

Contact Zones Among Lineages

Considering the highly differentiated nature of most lineages and the large geographic distances they cover, areas of potential contact among them may be useful in for future studies exploring whether or not reproductive isolation barriers exist among

lineages. As mentioned above, Barber found evidence of recent expansion of the CD lineage north of the Chiricahua mountains in central New Mexico and central Arizona. We suggest that expansion of the CD lineage led to secondary contact between the USA lineages. Our data also support introgression of the CD mitochondrial genome into the CP lineage from individuals sampled in this area of expansion, the Sierra Ancha region (Fig. 4-3). This is concordant with Barber's finding of mixed mitochondrial haplotypes of these different clades in a location adjacent to our sampling (1999a). Our results from the cluster analysis indicate other areas of potential recent gene flow. STRUCTURE results show individuals from the Pinaleños Mountains in the SD lineage sharing alleles with the CP/GC cluster. Supporting this evidence, Barber (1999b) inferred a recent range expansion/ colonization of the SD lineage into this area. Finally, individuals from the CD lineage (Chiricahua Mountains) share alleles with the SD cluster. Because of the geographically specificity of some of these shared allele occurrences, and because *H. arenicolor* lineages have high Fst values (except CP and GC), we believe this pattern is likely to be a result of secondary contact among previously isolated groups rather than incomplete lineage sorting.

General Conclusions

Our study reveals both previously undetected relationships and lack of phylogenetic resolution among lineages that appear to be incongruent with expected mtDNA relationships. We suggest that the mtDNA phylogeny is an inaccurate representation of the species phylogeny, and we feel that the AFLP data better reflect the main *H. arenicolor* lineage relationships. The AFLP data also suggest a history of isolation and subsequent secondary contact among lineages. Overall, AFLP data suggest

that canyon treefrog lineages diverged from a common ancestor more recently than predicted from mitochondrial data. If so, there are important consequences for interpreting studies of advertisement call evolution in this group. Before the discovery of mtDNA introgression and past hybridization with the *H. eximia* complex, the lack of call differences among populations of the canyon treefrog seemed counterintuitive to stochastic patterns of character divergence (Heyer & Reid, 2003), suggesting that selection could be acting to conserve call structure. However in the light of multi-locus data and multi-species sampling, call divergence appears to correspond to nuclear genetic divergence, which suggests that drift rather than selection is a major force acting on call divergence (Klymus et al., 2010). Furthermore, the recent studies of this system highlight the importance of sampling strategies in phylogeographic studies. As suggested by Funk and Omland (2003), sampling closely related congeneric species may reveal insights into seemingly paraphyletic relationships. Our results provide understanding into the complex evolutionary history of this group, have implications for the study of character evolution in this group, and emphasize the need for phylogeographic studies to expand sampling to include closely related, syntopic species.

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Table 4-1. Locality information for specimens examined.
The map symbols indicate the sampling locations in the map in Figure 4-1. The mtDNA lineage abbreviation represents the phylogeographic lineage assumed before phylogenetic analyses. Note only two specimens (indicated by *) for which we had mtDNA sequence were found to have a different mtDNA lineage than what they were assigned.

Species	Specimen	Location	Map Symbol	mtDNA Lineage	Latitude	Longitude	GenBank mtDNA
Hyla arenicolor	00001H	Peach Springs, Grand Canyon, AZ, USA	DC	GC	35.586	-113.435	GU989087
Hyla arenicolor	000B9H	Peach Springs, Grand Canyon, AZ, USA	DC	GC	35.586	-113.435	GU989076
Hyla arenicolor	00008H	Diamond Creek, Grand Canyon, AZ, USA	DC	GC	35.766	-113.373	GU989077
Hyla arenicolor	00099H	Diamond Creek, Grand Canyon, AZ, USA	DC	GC	35.766	-113.373	
Hyla arenicolor	00062H	Bridge Canyon, Grand Canon, AZ, USA	DS	GC	35.774	-113.523	
Hyla arenicolor	00063H	Spencer Canyon, Grand Canyon, AZ, USA	DS	GC	35.824	-113.648	GU989091
Hyla arenicolor	00064H	Salt Creek, Grand Canyon, AZ, USA	DS	GC	36.027	-113.783	
Hyla arenicolor	00065H	Cave Canyon, Grand Canyon, AZ, USA	DS	GC	36.096	-113.921	GU989092
Hyla arenicolor	00104H	Grapevine, Lake Mead, AZ, USA	DS	GC	36.045	-114.026	JN830875
Hyla arenicolor	00105H	Grapevine, Lake Mead, AZ, USA	DS	GC	36.045	-114.026	JN830874
Hyla arenicolor	00100H	Havasu Creek 1, Grand Canyon, AZ, USA	US	GC	36.282	-112.865	JN830878
Hyla arenicolor	00101H	Havasu Creek 1, Grand Canyon, AZ, USA	US	GC	36.282	-112.865	
Hyla arenicolor	00102H	Havasu Creek 2, Grand Canyon, AZ, USA	US	GC	36.281	-112.888	JN830873
Hyla arenicolor	00103H	Havasu Creek 2, Grand Canyon, AZ, USA	US	GC	36.281	-112.888	
Hyla arenicolor	00144H⌘	Kwagunt Creek, Grand Canyon, AZ, USA	KWC	GC	36.265	-111.828	
Hyla arenicolor	00145H⌘	Kwagunt Creek, Grand Canyon, AZ, USA	KWC	GC	36.265	-111.828	JN830877
Hyla arenicolor	00146H⌘	Kwagunt Creek, Grand Canyon, AZ, USA	KWC	GC	36.265	-111.828	JN830876
Hyla arenicolor	00012H	L. Workman Creek, Sierra Ancha Mountains, AZ, USA	SA	CP	33.846	-110.972	
Hyla arenicolor	00017H	Parker Canyon, Sierra Ancha Mountains, AZ, USA	SA	CP*	33.798	-110.967	JN830891
Hyla arenicolor	00018H	Parker Canyon, Sierra Ancha Mountains, AZ, USA	SA	CP	33.798	-110.967	
Hyla arenicolor	00A28H	Reynolds Creek, Sierra Ancha Mountains, AZ, USA	SA	CP	33.874	-110.992	
Hyla arenicolor	00049H	Houston Mesa Crossing, Payson, AZ, USA	SA	CP*	34.364	-111.282	GU989065
Hyla arenicolor	00050H	Pine Creek, Zion National Park, UT, USA	ZNP	CP	37.217	-112.974	GU989084
Hyla arenicolor	00066H	Oak Creek, Zion National Park, UT, USA	ZNP	CP	37.213	-112.994	JN830884
Hyla arenicolor	00068H	Keyhole, Zion National Park, UT, USA	ZNP	CP	37.225	-112.903	
Hyla arenicolor	00069H	Keyhole, Zion National Park, UT, USA	ZNP	CP	37.225	-112.903	
Hyla arenicolor	00070H¥	Coyote Gulch, UT, USA	CG	CP	37.426	-111.078	JN830886

Species	Specimen	Location	Map Symbol	mtDNA Lineage	Latitude	Longitude	GenBank mtDNA
Hyla arenicolor	00071H[¥]	Coyote Gulch, UT, USA	CG	CP	37.426	-111.078	
Hyla arenicolor	00072H[¥]	Coyote Gulch, UT, USA	CG	CP	37.426	-111.078	JN830887
Hyla arenicolor	00073H[¥]	Coyote Gulch, UT, USA	CG	CP	37.426	-111.078	
Hyla arenicolor	00089H[¥]	John Brown Canyon, CO, USA	JB	CP	38.638	-108.995	JN830885
Hyla arenicolor	00090H[¥]	John Brown Canyon, CO, USA	JB	CP	38.638	-108.995	
Hyla arenicolor	00091H[¥]	John Brown Canyon, CO, USA	JB	CP	38.638	-108.995	
Hyla arenicolor	00092H[¥]	John Brown Canyon, CO, USA	JB	CP	38.638	-108.995	
Hyla arenicolor	00096H	Wilhoit, AZ, USA	WL	CP	34.399	-112.536	JN830888
Hyla arenicolor	00097H	Wilhoit, AZ, USA	WL	CP	34.399	-112.536	
Hyla arenicolor	00098H	Wilhoit, AZ, USA	WL	CP	34.399	-112.536	
Hyla arenicolor	00121H	Wilhoit, AZ, USA	WL	CP	34.399	-112.536	
Hyla arenicolor	00138H	Kelly Canyon, AZ, USA	KC	CP	35.059	-111.717	JN830889
Hyla arenicolor	00139H	Kelly Canyon, AZ, USA	KC	CP	35.059	-111.717	
Hyla arenicolor	00149H	Kelly Canyon, AZ, USA	KC	CP	35.059	-111.717	
Hyla arenicolor	00142H	Kelly Canyon, AZ, USA	KC	CP	35.059	-111.717	
Hyla arenicolor	00051H	Chiricahua Mountains, AZ, USA	CH	CD	31.753	-109.418	GU989075
Hyla arenicolor	00075H	Chiricahua Mountains, AZ, USA	CH	CD	31.841	-109.278	JN830892
Hyla arenicolor	00076H	Chiricahua Mountains, AZ, USA	CH	CD	31.841	-109.278	
Hyla arenicolor	00077H	Chiricahua Mountains, AZ, USA	CH	CD	31.841	-109.278	
Hyla arenicolor	03468H[Ψ]	Davis Mountains, TX, USA	DM	CD	30.685	-104.078	GU989080
Hyla arenicolor	00093H[Φ]	Davis Mountains, TX, USA	DM	CD	30.814	-103.937	
Hyla arenicolor	00094H[Φ]	Davis Mountains, TX, USA	DM	CD	30.814	-103.937	
Hyla arenicolor	00079H[¥]	Davis Mountains, TX, USA	DM	CD	30.510	-103.747	
Hyla arenicolor	00080H[¥]	Davis Mountains, TX, USA	DM	CD	30.510	-103.747	
Hyla arenicolor	00052H	Jacobson's Creek, Pinaleño Mountains, AZ, USA	PM	SD	32.667	-109.803	GU989067
Hyla arenicolor	00081H	Jacobson's Creek, Pinaleño Mountains, AZ, USA	PM	SD	32.667	-109.803	
Hyla arenicolor	00082H	Jacobson's Creek, Pinaleño Mountains, AZ, USA	PM	SD	32.667	-109.803	

Species	Specimen	Location	Map Symbol	mtDNA Lineage	Latitude	Longitude	GenBank mtDNA
Hyla arenicolor	00083H	Jacobson's Creek, Pinaleño Mountains, AZ, USA	PM	SD	32.667	-109.803	
Hyla arenicolor	00084H	Jacobson's Creek, Pinaleño Mountains, AZ, USA	PM	SD	32.667	-109.803	
Hyla arenicolor	00A20H	Box Canyon, Santa Rita Mountains, AZ, USA	SR	SD	31.798	-110.777	GU989072
Hyla arenicolor	00B43H	Santa Rita Field Station, Santa Rita Mountains, AZ, USA	SR	SD	31.739	-110.866	GU989066
Hyla arenicolor	00085H	Madera Canyon, Santa Rita Mountains, AZ, USA	SR	SD	31.735	-110.883	
Hyla arenicolor	00095H	Cave Canyon, Santa Rita Mountains, AZ, USA	SR	SD	31.709	-110.771	
Hyla arenicolor	00046H	Gardner Canyon, Santa Rita Mountains, AZ, USA	SR	SD	31.709	-110.771	
Hyla arenicolor	00053H	Ruby Road, Tumacacori Mountains, AZ, USA	RR	SD	31.397	-111.139	GU989086
Hyla arenicolor	00086H	Ruby Road, Tumacacori Mountains, AZ, USA	RR	SD	31.397	-111.139	
Hyla arenicolor	00127H	Scotia Canyon, Huachuca Mountains, AZ, USA	SC	SD	31.465	-110.462	
Hyla arenicolor	00041H$^{\Psi}$	Huajolotitlan, Oaxaca, MX	BAL	BAL	17.815	-97.741	JN830870
Hyla arenicolor	00044H$^{\Psi}$	Huajuapan de Leon, Oaxaca, MX	BAL	BAL	17.805	-97.781	JN830871
Hyla arenicolor	001HNH$^{\Psi}$	Huajuapan de Leon, Oaxaca, MX	BAL	BAL	17.805	-97.781	JN830869
Hyla arenicolor	0MX29H$^{\Omega}$	Corral de Piedra, Oaxaca, MX	BAL	BAL	17.754	-97.737	
Hyla arenicolor	0MX31H$^{\Omega}$	Calatepec, Puebla, MX	BAL	BAL	18.189	-97.483	
Hyla arenicolor	00054H$^{\Psi}$	Jocotepec, Jalisco, MX	JAL	CMP-SW	20.309	-103.445	JN830880
Hyla arenicolor	00055H$^{\Psi}$	Jalisco, Bosque de La Primavera, Jalisco,MX	JAL	CMP-SW	20.596	-103.639	JN830881
Hyla arenicolor	00056H$^{\Psi}$	Jocotepec, Jalisco, MX	JAL	CMP-SW	20.309	-103.445	JN830882
Hyla arenicolor	00057H$^{\Psi}$	Jocotepec, Jalisco, MX	JAL	CMP-SW	20.309	-103.445	JN830883
Hyla arenicolor	0MX32H$^{\Omega}$	Jiquilipan, Michoacán, MX	JAL	CMP-SW	19.956	-102.804	
Hyla arenicolor	0MX28H$^{\Psi}$	Cerro Colorado, Guanajuato, MX	GTO	CMP	20.232	-100.347	JN830890
Hyla wrightorum	00058H	Peterson's Ranch, Huachuca Mountains, AZ, USA	HUA		31.494	-110.403	GU989085
Hyla wrightorum	00126H$^{¥}$	Scotia Canyon, Huachuca Mountains, AZ, USA	HUA		31.465	-110.463	
Hyla wrightorum	00115H$^{¥}$	Scotia Canyon, Huachuca Mountains, AZ, USA	HUA		31.434	-110.418	
Hyla wrightorum	00116H$^{¥}$	Scotia Canyon, Huachuca Mountains, AZ, USA	HUA		31.434	-110.418	
Hyla wrightorum	00117H$^{¥}$	Scotia Canyon, Huachuca Mountains, AZ, USA	HUA		31.434	-110.418	
Hyla wrightorum	00118H$^{¥}$	Scotia Canyon, Huachuca Mountains, AZ, USA	HUA		31.434	-110.418	

Species	Specimen	Location	Map Symbol	mtDNA Lineage	Latitude	Longitude	GenBank mtDNA
Hyla wrightorum	00059H	Gila Co., AZ , USA	CAZ2		34.287	-110.858	GU989069
Hyla wrightorum	00060H	Gila Co., AZ , USA	CAZ2		34.253	-110.844	GU989089
Hyla wrightorum	00124H	Springerville, AZ, USA	CAZ3		34.055	-109.353	JN830872
Hyla wrightorum	00125H	Springerville, AZ, USA	CAZ3		34.055	-109.353	
Hyla wrightorum	00136H	Kelly Canyon, AZ, USA	CAZ4		35.059	-111.717	
Hyla wrightorum	00112H¥	Coconino Co., AZ, USA	CAZ4		35.262	-111.830	
Hyla wrightorum	00113H¥	Coconino Co., AZ, USA	CAZ4		35.262	-111.830	
Hyla wrightorum	00114H¥	Coconino Co., AZ, USA	CAZ4		35.262	-111.830	
Hyla eximia	00370Hψ	Cuquio, Jalisco, MX	CUI		20.917	-103.033	GU989083
Hyla eximia	00006Hψ	road to Concepcion de Buenos Aires, Jalisco, MX	CON		19.967	-103.267	JN830879
Hyla eximia	00119Hψ	road to Concepcion de Buenos Aires, Jalisco, MX	CON		19.967	-103.267	
Hyla eximia	00120Hψ	road to Concepcion de Buenos Aires, Jalisco, MX	CON		19.967	-103.267	
Hyla femoralis	03858Hψ	Chatham Co., GA, USA			31.999	-81.120	GU989073
Hyla femoralis	00133H	Apalachicola National Forest, FL, USA			29.861	-84.986	
Hyla femoralis	00134H	Apalachicola National Forest, FL, USA			29.861	-84.986	
Hyla femoralis	00135H	Apalachicola National Forest, FL, USA			29.861	-84.986	
Hyla chrysoscelis	00129H	Phelps Co., MO, USA			37.609	-91.976	
Hyla chrysoscelis	00061H	Phelps Co., MO, USA			37.609	-91.976	GU989082
Hyla chrysoscelis	00128H	Webb Wildlife Management, SC, USA			32.577	-81.315	
Hyla chrysoscelis	00131H	Webb Wildlife Management, SC, USA			32.577	-81.315	

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Ω Museo de Zoología Alfonso L. Herrera, Facultad de Ciencias, Universidad Nacional Autónoma de México, Distrito Federal, México

Table 4-2. Primers used for amplification of mtDNA fragments.
Source indicates original study in which primers were designed. Superscript letters designate primer pairs.

Primer Name	Primer Sequence (5'-3')	Source
MVZ-59[a]	ATAGCACTGAAAAYGCTDAGATG	(Goebel et al., 1999)
tRNAVal[a]	GGTGTAAGCGARAGCTTTKGTTAAG	(Goebel et al., 1999)
12L1[b]	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	(Goebel et al., 1999)
16Sh[b]	GCTAGACCATKATGCAAAAGGTA	(Goebel et al., 1999)
12Sm[c]	GGCAAGTCGTAACATGGTAAG	(Pauly et al., 2004)
16Sa[c]	ATGTTTTTGGTAAACAGGCG	(Goebel et al., 1999)
16Sc[d]	GTRGGCCTAAAAGCAGCCAC	(Pauly et al., 2004)
16Sd[d]	CTCCGGTCTGAACTCAGATCACTGAG	(Pauly et al., 2004)

Table 4-3. Pairwise p-distance values among lineages based on mtDNA sequence.

	HEX	HWR	BAL	CP	GC	SD	CD	CMP-SW	HFE
HEX	----								
HWR	0.024	----							
BAL	0.041	0.043	----						
CP	0.046	0.042	0.059	----					
GC	0.025	0.002	0.042	0.042	----				
SD	0.046	0.042	0.058	0.035	0.043	----			
CD	0.044	0.041	0.059	0.009	0.041	0.032	----		
CMP-SW	0.022	0.026	0.047	0.047	0.026	0.050	0.045	----	
HFE	0.068	0.062	0.077	0.066	0.062	0.063	0.064	0.066	----
HCH	0.053	0.050	0.061	0.052	0.050	0.052	0.051	0.053	0.060

Table 4-4. Genetic diversity data for the lineages of *H. arenicolor* and the sister species, *H. wrightorum* (HWR) and *H. eximia* (HEX).
Note that the one CMP individual is included with the 9 CD individuals. Also, CP and GC combined shared 1 private allele. H_e is the expected heterozygosity under Hardy-Weinberg, also known as Nei's genetic diversity. SE H_e is the standard error of H_e.

Lineage	n	number of loci	% polymorphic loci	number of private alleles	H_e	SE H_e
HEX	4	727	49.5	1	0.22595	0.00736
HWR	14	727	40.4	6	0.13439	0.00642
BAL	5	727	36.5	20	0.10321	0.00570
CP	25	727	18.8	0	0.08556	0.00545
GC	17	727	14.9	0	0.0764	0.00532
SD	13	727	43.9	1	0.13211	0.00614
CD (CMP)	10	727	49.4	0	0.15921	0.00654
CMP-SW	5	727	46.1	1	0.16425	0.00656

Table 4-5. Pairwise Nei's genetic distances are shown below the diagonal and pairwise Fst values above the diagonal for the six *H. arenicolor* lineages (BAL, CD, CP, GC, CMP-SW, and SD) and *H. wrightorum* (HWR) and *H. eximia* (HEX).

	HEX	HWR	BAL	CP	GC	SD	CD	CMP-SW
HEX	0	0.3166	0.4767	0.5285	0.5502	0.4308	0.3446	0.3409
HWR	0.1058	0	0.6410	0.6642	0.6814	0.5734	0.4946	0.4908
BAL	0.195	0.2751	0	0.7292	0.7484	0.6487	0.6019	0.6093
CP	0.2281	0.2797	0.3290	0	0.0458	0.4879	0.4927	0.5322
GC	0.2417	0.2896	0.3471	0.0042	0	0.5113	0.5127	0.5488
SD	0.1786	0.2314	0.2824	0.1233	0.1293	0	0.3976	0.4184
CD	0.1331	0.1842	0.2585	0.1446	0.1502	0.1192	0	0.3343
CMP-SW	0.1332	0.1851	0.2747	0.1761	0.1806	0.1334	0.1019	0

Table 4-6. The average LnP(D) values from STRUCTURE and the Δ K values as calculated by the method of Evanno et al. (2005).

K	average LnP (D)	Δ K
1	-37177.71	-------
2	-27338.43	269.5
3	-23838.10	41.0
4	-21575.70	0.6
5	-19106.81	24.5
6	-18426.96	0.8
7	-17279.19	295.6
8	-30050.33	1.9
9	-28126.50	1.7
10	-30233.65	1.1

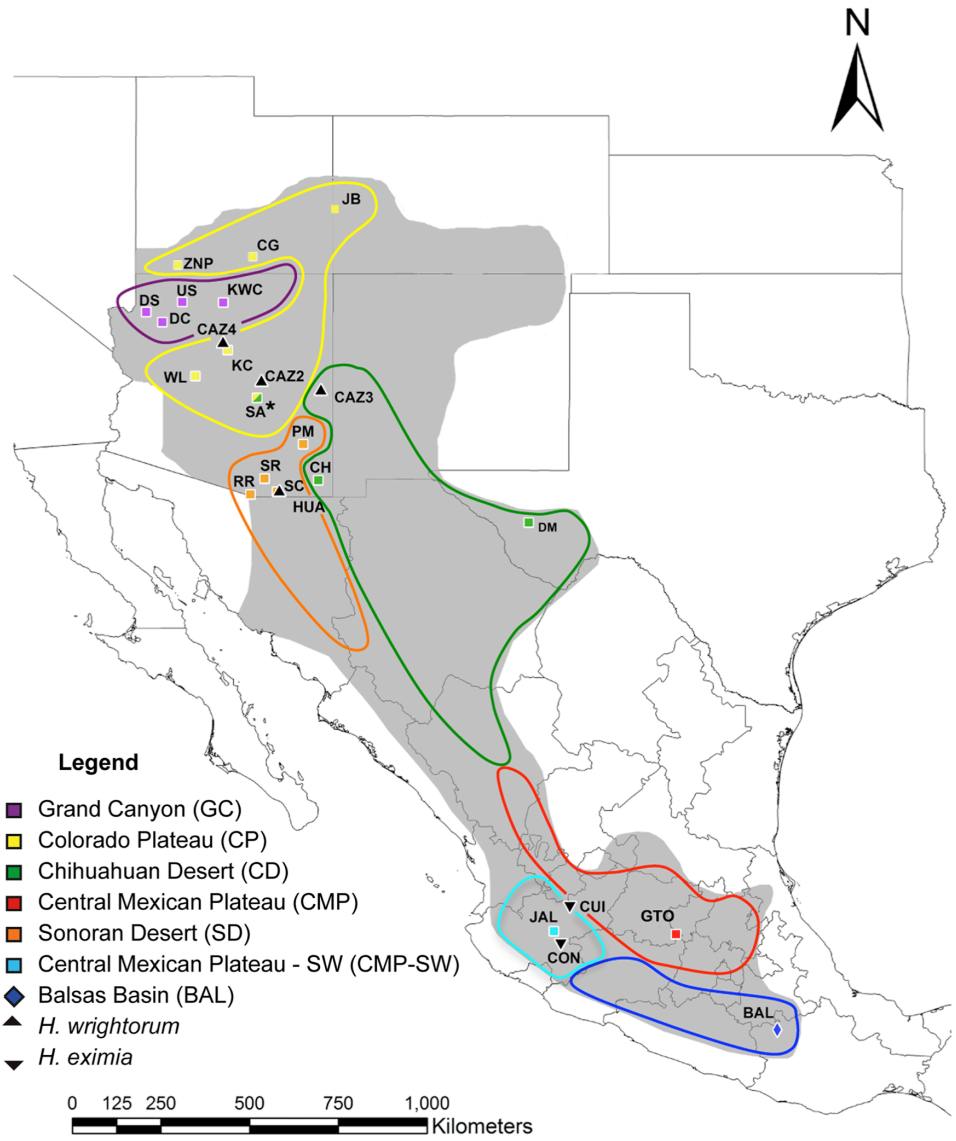

Figure 4-1. Map showing the range of *Hyla arenicolor* in gray and our sampling throughout the seven phylogeographic lineages, along with our sampling of *H. wrightorum* and *H. eximia*.
Hypothetical phylogeographic lineage ranges from Bryson et al (2010) are outlined in colors, and the symbol denoting each sampling locality for *H. arenicolor* is similarly colored. Note the that the Sierra Ancha locality (SA*) represents a contact zone between the CP and CD lineages, with some samples having the CD mtDNA haplotype with a CP nuclear (AFLP) background.

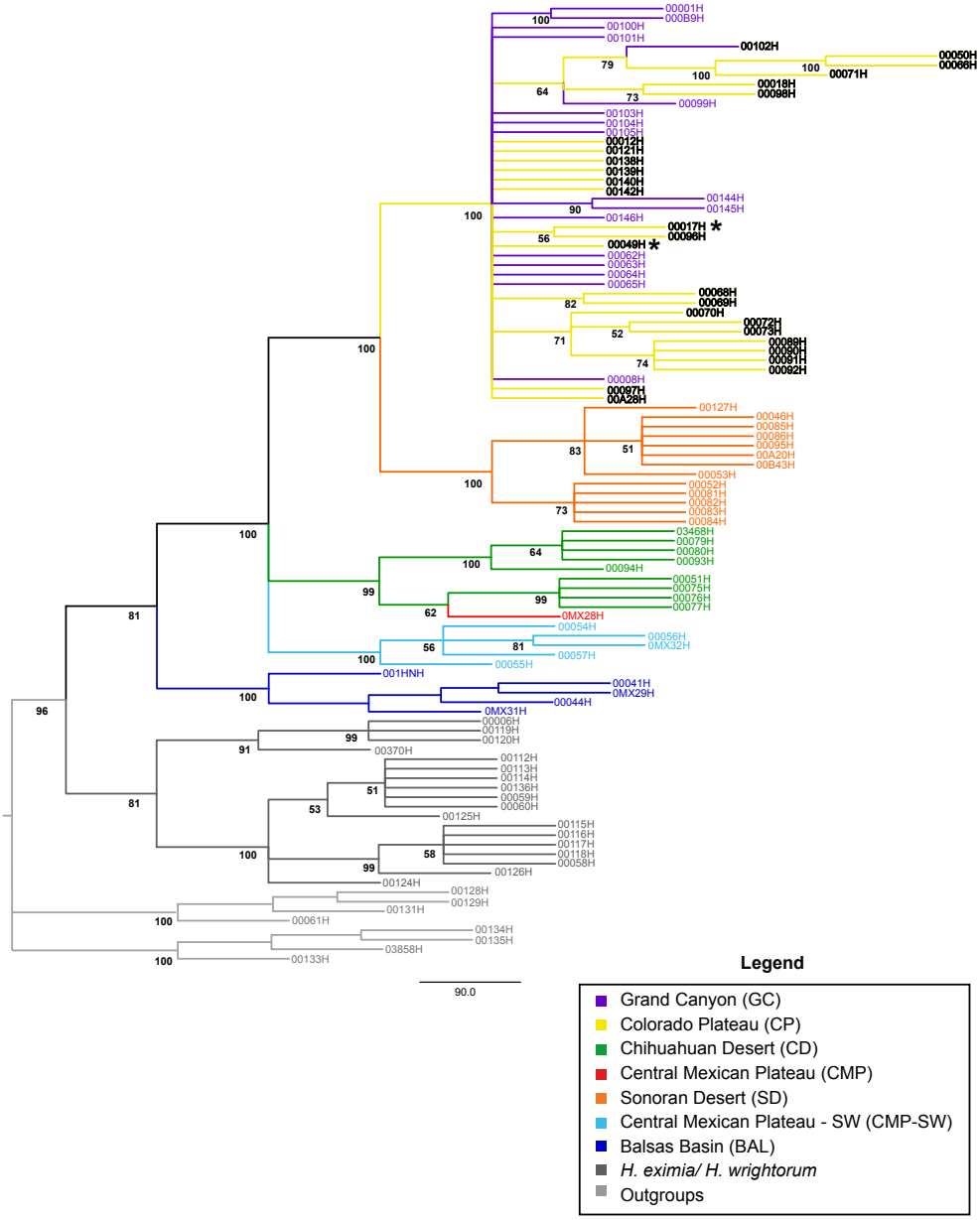

Figure 4-2. Neighbor-joining phenogram of *H. arenicolor* lineages, the BAL lineage, *H. wrightorum, H. eximia*, and two outgroup species based on 727 AFLP characters. Colors of branches represent the phylogeographic lineage from which the individuals were sampled. Samples with the * represent individuals that have the mtDNA haplotype of the CD lineage, but have the AFLP nuclear background of the CP lineage. Support values based on 2000 bootstrap replicates are shown at nodes.

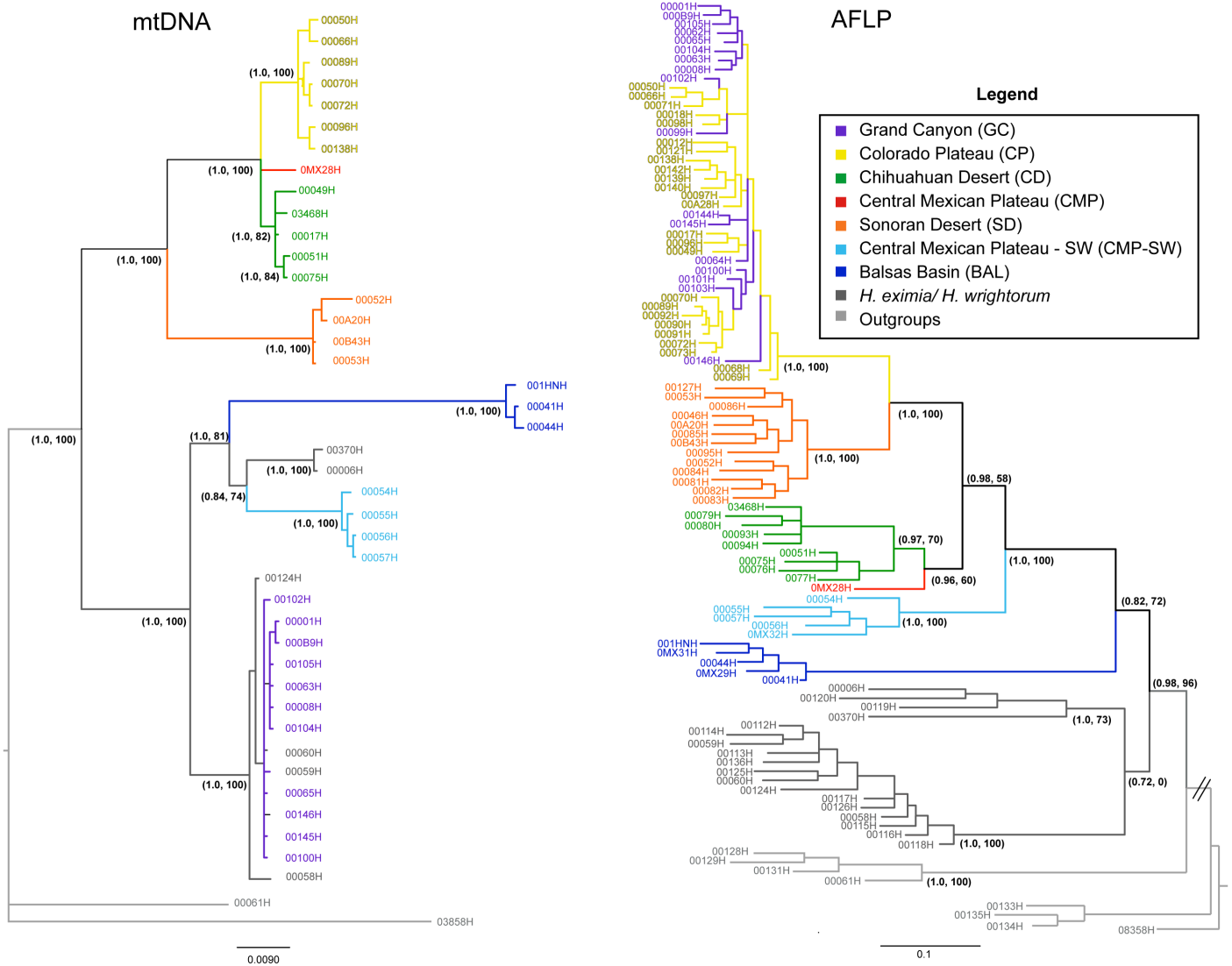
mtDNA
00050H
00066H
00089H
00070H
00072H
00096H
00138H
0MX28H
00049H
03468H
00017H
00051H
00075H
00052H
00A20H
00B43H
00053H
001HNH
00041H
00044H
00370H
00006H
00054H
00055H
00056H
00057H
00124H
00102H
00001H
000B9H
00105H
00063H
00008H
00104H
00060H
00059H
00065H
00146H
00145H
00100H
00058H
00061H
03858H
0.0090
AFLP
Legend
Grand Canyon (GC)
Colorado Plateau (CP)
Chihuahuan Desert (CD)
Central Mexican Plateau (CMP)
Sonoran Desert (SD)
Central Mexican Plateau - SW (CMP-SW)
Balsas Basin (BAL)
H. eximia/ H. wrightorum
Outgroups
0.1

Figure 4-3. Bayesian inferred phylogenies using mtDNA sequence and AFLP data. Support values (Bayesian posterior probabilities and parsimony bootstrap) are in parentheses. Note the well-supported sister relationship between the SD and CP/GC lineages in the AFLP topology.

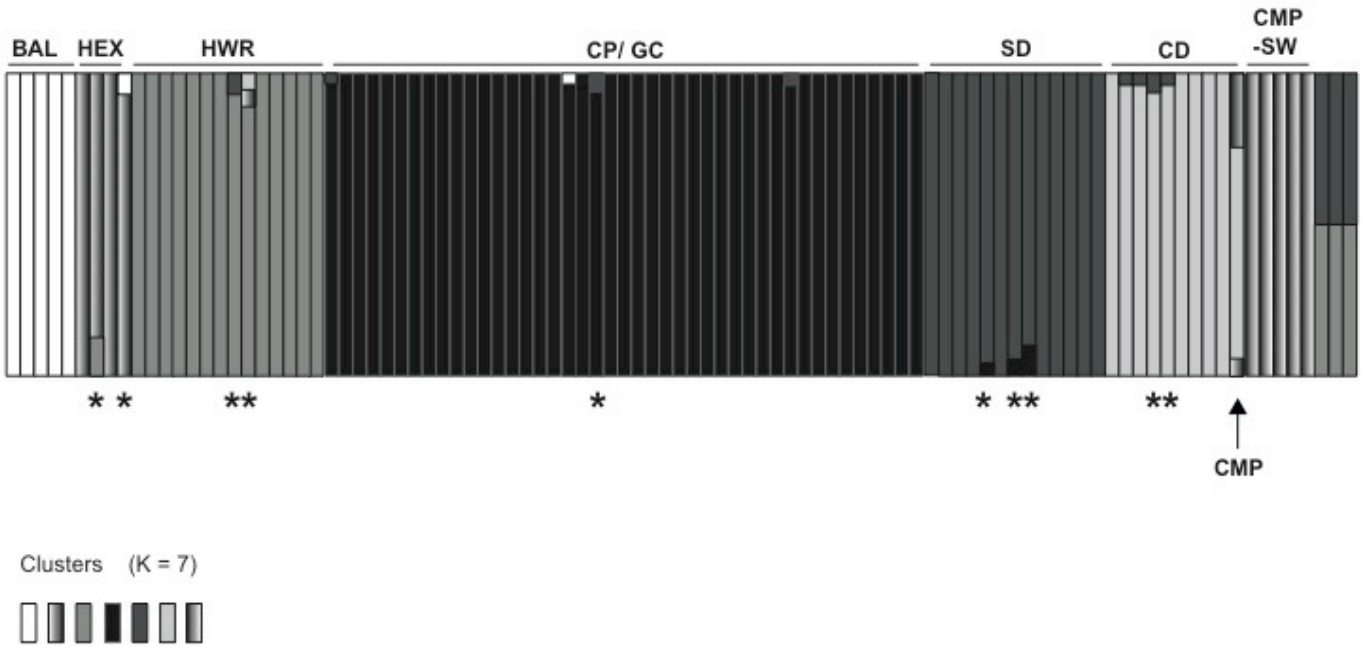

Figure 4-4. Results from STRUCTURE cluster analysis.
Of the eight lineages (6-*H.arenicolor*, 1- *H.eximia*, 1-*H.wrightorum*) surveyed, seven clusters (different gray tones) were inferred (with the CP and GC lineages in one cluster). Samples with * represent individuals that shared alleles with other clusters throughout all ten runs. The single CMP individual grouped with the CD lineage, but shows allele sharing with the CMP-SW and *H. eximia*. The last three individuals are artificially crossed hybrids of the *H. arenicolor* SD lineage and *H. wrightorum*.

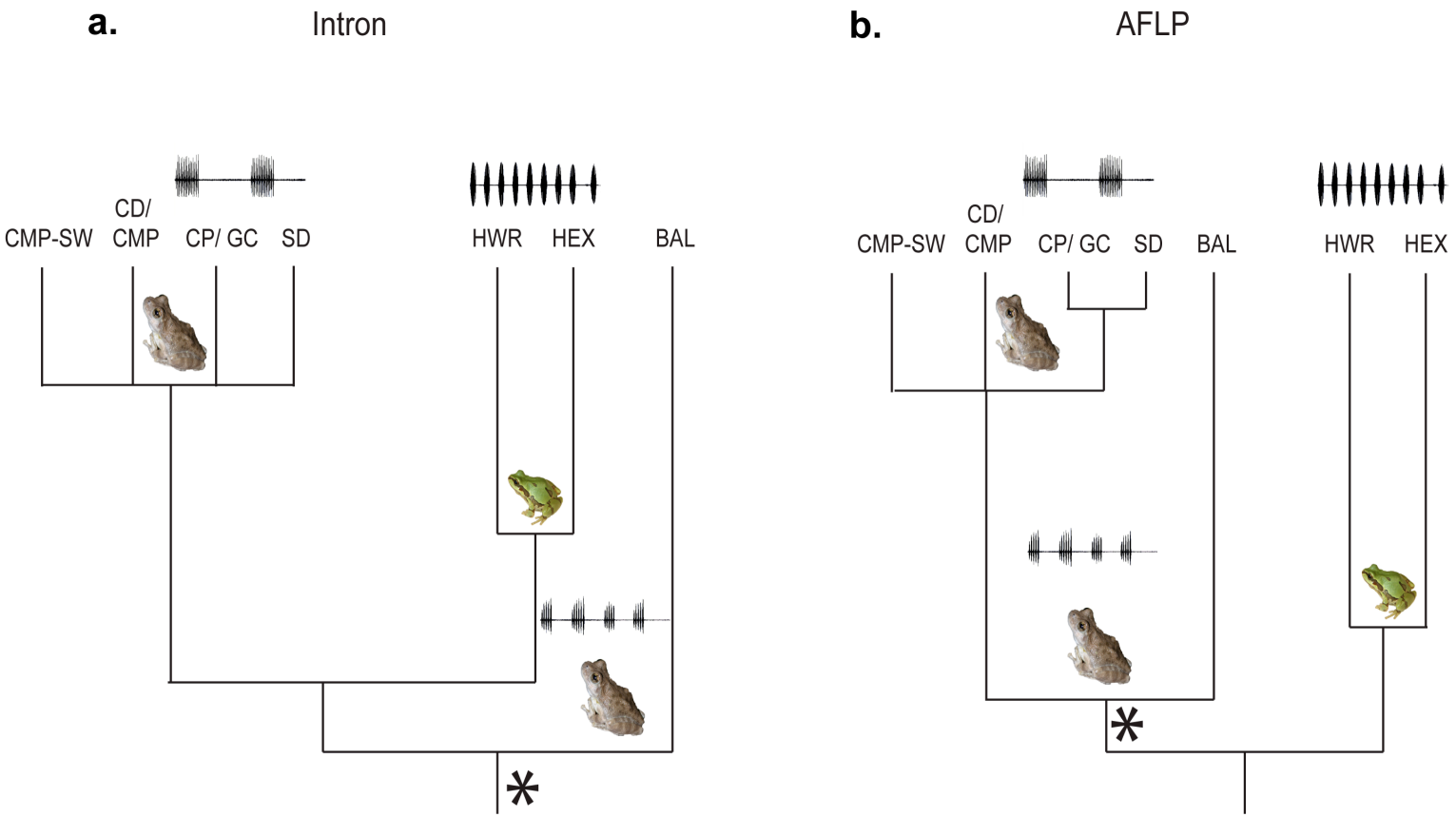

Figure 4-5. A diagram showing the inferred phylogenetic relationships of the *H. arenicolor* lineages and the sister *H. eximia* complex. (a) Relationships based on intron data from Bryson et al. (2010). (b) Relationships based on phylogenetic inference from AFLP data in this study. Images representative of these frogs and waveforms of their advertisement calls are included at nodes. The asterisk denotes the unresolved placement of the BAL lineage.

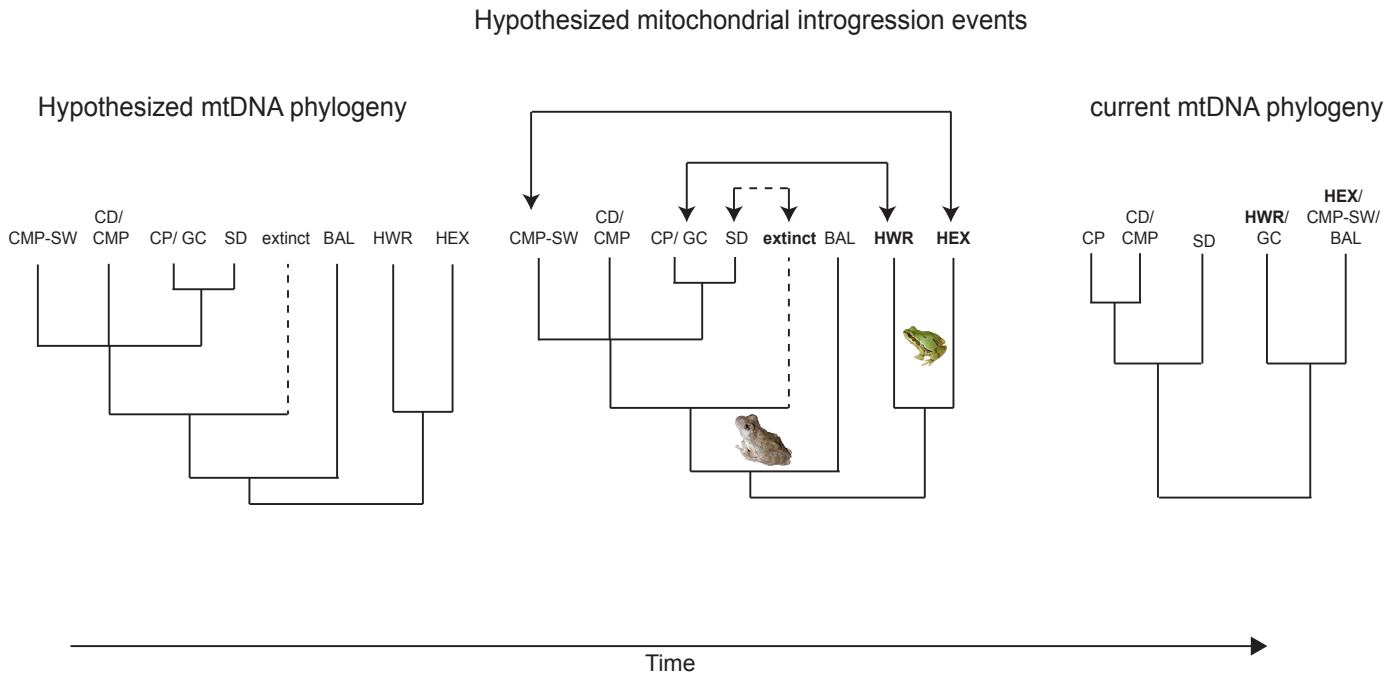

Figure 4-6. Depiction of the proposed scenario in which the SD clade hybridized with an extinct lineage, resulting in the introgression of that extinct lineage's mitochondrial genome into the SD clade.
The tree on the left represents a hypothetical phylogeny prior to introgression. The center tree shows the introgression events between lineages connected by arows. This hypothesized introgressive event between the SD and extinct lienages is shown with the dotted arrows. Inferred introgressive events between *H. arenicolor* and the *H. eximia* complex are represented by the sold arrows. The tree on the right depicts the current phylogeny as inferred with mtDNA. Lineages in bold represent the groups whose mitochnodrial genome was introgressed into other lineages.

CHAPTER 5 CONCLUSION

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Studies of speciation that focus on examining current population differentiation within a species predict a general increase in phenotypic divergence with increasing genetic and geographic distances. Such an observation is expected because of isolation by distance effects on gene flow and subsequent effects of genetic drift on trait divergence (Emerson & Ward, 1998; Panhuis et al., 2001; Ruegg et al., 2006). When this prediction is not observed, selection is believed to play a strong role in the divergence or conservation of a trait (Ryan et al., 1996; Masta & Maddison, 2002; Pröhl et al., 2006; Irwin et al., 2008). Studies of secondary sexual traits are of particular interest as they may be influenced by sexual selection, which can lead to rapid trait divergence (Panhuis et al., 2001; Questiau, 1999). By studying the correspondence between genetic and phenotypic differences we can better understand the roles that selection and drift play in driving speciation (Hankison & Ptacek, 2008).

My dissertation aimed to understand patterns of differentiation in a secondary sexual trait, male advertisement calls, among populations of a widespread species, the canyon treefrog, *Hyla arenicolor*. The first study in my dissertation addressed whether selection acted to conserve advertisement call structure as populations from the USA portion of the range were found to exhibit very little call differentiation despite large mtDNA genetic divergences (Chapter 2). However, the study's focus shifted after re-examining the original phylogenetic inference which suggested that the Grand Canyon (GC) lineage was more closely related to the outgroup species group, *H. eximia*/ *H. wrightorum* (Barber, 1999). Using both nuclear and mtDNA sequences data, this study found that the Grand Canyon lineage actually has the introgressed mtDNA genome of the sister species group. Furthermore the study found that call divergence better reflected the

inferred nuclear phylogeny than that based on mtDNA. The Grand Canyon (GC) and Colorado Plateau (CP) lineages were found to have the least amount of call differentiation, were the most closely related based on nuclear sequence, and were geographically closest to one another relative to the other USA lineage (Sonoran Desert-SD). In fact nuclear sequence data could not resolve any difference between the GC lineage and the surrounding CP lineage. Thus the pattern between nuclear genetic and phenotypic divergence was different from that detected by the mtDNA data. The observed correspondence between nuclear genetic and advertisement call divergence thus suggests that drift, and not selection, has played a stronger role in phenotypic differentiation among populations. Call differences were observed between this combined CP/GC group and the SD lineage in southern Arizona. Although statistically significant, the magnitude of the call differences was thought to not be biologically relevant in terms of female preference.

An expanded study of call variation and female preference provided evidence to support the above prediction (Chapter 3). Data from playback experiments showed that females from the combined CP/GC lineage do not show a preference for their call type over that of the neighboring SD lineage. Thus if theses lineages do come into contact, call differentiation would be insufficient to lead to behavioral reproductive isolation through call discrimination. This research also quantified biologically significant differences thoughout the species' range in pulse rate, call duration and call period. Females from the combined CP/GC lineage were able to discriminate against the natural calls of two of the Mexican lineages using differences in pulse rate, call duration and call period. As previously suggested by Pierce (1968), the Balsas Basin (BAL) lineage of Mexico was

found to have the most divergent call type. Besides the large call duration and call period differences already described by Pierce (1968), our study found that calls from the BAL lineage also have a lower pulse rate. Interestingly, the neighboring lineages of the Central Mexican Plateau, CMP and CMP-SW, have a pulse rate higher than both USA lineages and the BAL lineage. Future studies would benefit from increased sampling of calls in all the Mexican lineages and the Chihuahuan Desert lineage, as well as testing female preference among the other lineages.

Finally, my dissertation assessed phylogenetic relationships among the *H. arenicolor* lineages using AFLP (amplified fragment length polymorphism) molecular markers. Since the introduction of the method by Vos et al. (1995), such markers have been used extensively in phylogenetic inference in plants, and their use in animal studies is growing (Meudt & Clark, 2007). These studies suggest that AFLP markers provide phylogenetic signal especially in shallow divergences that may be difficult to assess with slower evolving sequence data (Koopman, 2005). The canyon treefrog is an excellent candidate for such a study, as mitochondrial introgression precludes the use of mtDNA markers. Furthermore, a study using intron sequence to define phylogeographic structure in the canyon treefrog was unable to resolve the relationships among the known lineages (Bryson et al., 2010). My study (Chapter 4) resolved relationships among some *H. arenicolor* lineages. However it was unable to resolve the relation between the CP and GC lineages, suggesting that extensive nuclear gene flow occurs between these lineages despite the geographical localization of highly divergent mitochondrial haplotypes. Results also show that the BAL lineage is highly divergent from all other *H. arenicolor* lineages. The genetic and behavioral differences observed in this study indicate that the

BAL lineage likely represents a new species. Finally, results from population structure analysis also reveals that genetic divergences among the lineages are very high (with the exception of the CP/GC group), suggesting they have been separated through allopatry for a long period.

The role of drift versus selection in population divergence has been addressed in this system; however, because of limited call sampling throughout the Mexican portion of the range, measures of call distance were not calculated. Thus we cannot directly compare genetic, geographic and phenotypic (call) distances in order to ascertain the roles of evolutionary forces in this system. Nevertheless, overall patterns clearly demonstrate a correspondence among these measurements, suggesting neutral genetic drift has played a role in call divergence throughout most of the range. An exception to this pattern may be confirmed, however, by investigating the roles of drift and selection in the call divergence observed among the three Mexican lineages, as the CMP and CMP-SW show the largest degree of call divergence relative to the neighboring BAL lineage.

Another major conclusion from this work is the importance of sampling closely related and syntopic species in phylogenetic and phylogeographic studies. My research reveals a high amount of introgressive hybridization inferred between *H. arenicolor* and *H. eximia*/ *H. wrightorum*, as well as among *H. arenicolor* clades. Introgression of cytoplasmic markers is common and has been well documented in both plant and animal taxa (Dowling & Secor, 1997; Shaw, 2002; Baack & Rieseberg, 2007). This works emphasizes the need for multi-locus and multi-taxon studies. The degree of introgression in this system also raises the question of why these species appear to be so susceptible to mitochondrial introgression. If the introgressed mitochondrial genome is better adapted to

the local conditions of the population, then selection can fix this mitochondrial type (Ballard & Whitlock,2004). Changes in population demographics may also lead to an increased likelihood of mitochondrial introgression. The canyon treefrog may be prone to rapid population contractions and expansions, as they are restricted to mesic habitat surrounded by large expanses of desert. During range contractions, small populations that hybridized with *H. eximia*/ *H. wrightorum* may become fixed for the introgressed mitochondrial genome due to random genetic drift (Ballard & Whitlock, 2004). Further work exploring these hypotheses is needed.

My dissertation is the first study of the canyon treefrog to incorporate both behavioral and phylogenetic methods to address population differentiation. Previous work focused primarily on either behavior (acoustic: Pierce, 1968; temperature and water regulation: Wylie, 1981; Preest et al., 1992) or genetic differentiation (Murray, 1997; Barber, 1999; Bryson et al., 2010). By combining both methods, this dissertation provides insight into speciation by analyzing phenotypic divergence among populations. Whether or not this differentiation leads to actual speciation depends on the strength of the acting evolutionary forces. By comparing phenotypic traits (especially those important in reproductive isolation) with neutral genetic markers we can infer the strength of these factors (Ryan et al., 1996; Masta & Maddison, 2002; Swallow et al., 2005). Conclusions from my dissertation provide support for the hypothesis that drift has played a major role in call divergence throughout the range of the canyon treefrog. However, future work addressing female preference in potential contact zones among the Mexican lineages may be fruitful in detecting a role for selection. Call divergence in the Chihuahuan lineage also needs to be addressed, because this group connects the divergent

call types of the USA and Mexican lineages. Finally, my dissertation reveals a complex evolutionary history not just among canyon treefrog lineages, but also with the sister species group, *H. eximia*/ *H. wrightorum*. My work underscores the importance of incorporating multiple methods (behavior, genetics), multiple loci (nuclear and mtDNA), and multiple species (*H. arenicolor*, *H. eximia*/ H. *wrightorum*) for a more complete understanding of a species' evolutionary history and future.

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APPENDIX A: POSITION OF MTDNA PRIMERS

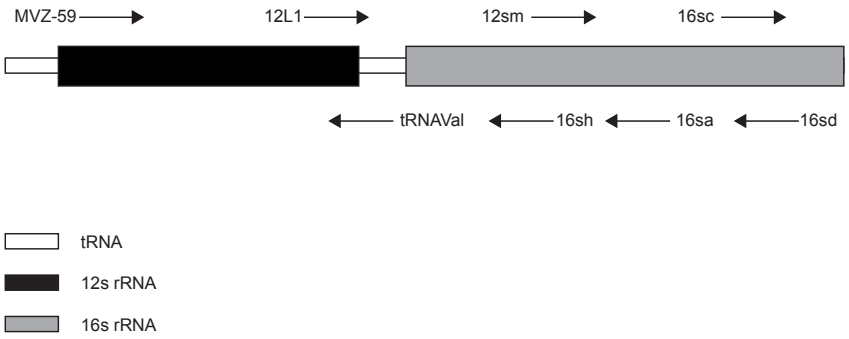

Figure A-1. Position of mitochondrial rRNA primers.

APPENDIX B: DESCRIPTION OF TEMPORAL CALL PROPERTIES MEASURED IN CHAPTER 3

Call Duration: measured from beginning of call to end of call; measured in milliseconds

Call Period: measured from the beginning of one call to the end of another call; measured in milliseconds

Pulse Rate: the inverse of pulse period, pulse period being measured from the beginning of one pulse to the beginning of the next; measured in pulses per second

Pulse Duration: measured from the beginning of one pulse to the end of the pulse; measured in milliseconds

Inter-Pulse Interval: measured as the interval between adjacent pulses; measured in milliseconds

Sub-Pulse Period: measured from the beginning of one sub-pulse to the beginning of another sub-pulse; measured in milliseconds

Call Duty Cycle: the amount of acoustic energy produced during one call bout, measured as the duration of the call relative to the duration of the call period; measured as a percentage of the call period

(Percentage) Pulse Rise Time: pulse rise time is measured as the time from the beginning of the pulse to its peak (highest acoustic energy); percentage pulse rise time is measured as a percentage of pulse duration

APPENDIX C: CALL TRAITS ANALYZED IN CHAPTER 3

Table C-1. Mean, standard deviation and ranges for call traits analyzed in Chapter 3.

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
High Frequency Peak (Hz)	23	CMP-SW	23	2779.44	275.099	1871.00-3180.00
		US-CP	45	2227.95	241.416	1616.00-2707.00
		US-GC	69	2295.97	356.858	1105.00-3070.00
		US-SD	36	2590.44	198.176	2138.00-3054.00
	20	BAL	8	2289.50	118.829	2079.00-2420.00
		US-CP	45	2115.13	241.416	1503.00-2594.00
		US-GC	69	2183.14	356.858	992.00-2957.00
		US-SD	36	2477.61	198.176	2025.00-2941.00
	16	CMP	12	2357.23	148.060	2063.00-2627.00
		US-CP	45	1964.70	241.416	1353.00-2444.00
		US -GC	69	2032.71	356.858	841.60-2806.00
		US-SD	36	2327.18	198.176	1875.00-2791.00
	18	US-CP	45	2040.00	241.416	1428.00-2519.0
		US-GC	69	2108.00	356.858	916.80-2882.00
		US-SD	36	2402.00	198.176	1950.00-2866.00
		CD	4	2083.02	309.23	1640.00-2325.02

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
Low Frequency Peak (Hz)	23	CMP-SW	23	665.13	74.250	563.00-865.00
		US-CP	45	596.36	66.308	506.80-819.40
		US -GC	69	616.89	48.050	534.90-770.60
		US-SD	36	677.04	68.900	570.10-852.80
	20	BAL	8	655.75	32.649	607.00-692.00
		US-CP	45	562.36	66.307	472.80-785.40
		US-GC	69	582.90	48.054	500.90-736.60
		US-SD	36	643.04	68.901	536.10-818.80
	16	CMP	12	547.52	35.812	471.00-610.40
		US-CP	45	517.04	66.308	427.50-740.10
		US-GC	69	537.57	48.054	455.60-691.30
		US-SD	36	597.71	68.901	490.80-773.50
	18	US-CP	45	539.70	66.308	450.10-762.70
		US-GC	69	560.20	48.054	478.30-713.90
		US-SD	36	620.40	68.900	513.50-796.10
		CD	4	573.68	30.40	544.07-615.53

Table C-1. Continued

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
Call duration (sec)	23	CMP-SW	23	0.662	0.180	0.474-1.234
		US-CP	40	0.842	0.271	0.447-1.794
		US-GC	71	0.622	0.211	0.259-1.661
		US-SD	33	0.590	0.195	0.210-1.071
	20	BAL	8	0.395	0.053	0.339-0.478
		US-CP	40	1.075	0.271	0.680-2.027
		US-GC	71	0.855	0.210	0.492-1.894
		US-SD	33	0.823	0.195	0.443-1.304
	16	CMP	12	1.104	0.169	0.856-1.384
		US-CP	40	1.386	0.271	0.990-2.338
		US-GC	71	1.166	0.210	0.803-2.205
		US-SD	33	1.134	0.195	0.754-1.615
	18	US-CP	40	1.231	0.271	0.835-2.183
		US-GC	71	1.011	0.210	0.647 -2.049
		US-SD	33	0.978	0.196	0.598-1.460
		CD	4	1.315	0.282	1.038-1.663

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
Call Period (sec)	23	CMP-SW	22	2.095	0.598	1.393-3.944
		US-CP	37	2.493	0.836	0.953-4.997
		US-GC	59	2.462	0.813	1.063-5.783
		US-SD	24	1.681	0.441	0.865-2.709
	20	BAL	8	0.859	0.162	0.600-1.158
		US-CP	37	3.167	0.836	1.626-5.671
		US-GC	59	3.135	0.813	1.737-6.457
		US-SD	24	2.355	0.441	1.539-3.383
	16	CMP	11	3.362	0.758	2.366-5.079
		US -CP	37	4.066	0.836	2.526-6.570
		US -GC	59	4.035	0.813	2.637-7.356
		US -SD	24	3.254	0.441	2.439-4.282
	18	US-CP	37	3.617	0.836	2.077-6.121
		US-GC	59	3.586	0.813	2.187-6.907
		US-SD	24	2.805	0.441	1.989-3.833
		CD	2	3.466	0.789	2.908-4.024

Table C-1. Continued

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
Pulse Rate (pulses / sec)	23	CMP-SW	23	27.74	1.891	23.33-31.07
		US-CP	44	21.88	1.076	19.93-24.15
		US -GC	71	21.13	0.883	18.85-22.95
		US-SD	38	20.66	0.969	18.18-22.42
	20	BAL	8	11.67	1.745	9.60-14.04
		US-CP	44	18.58	1.076	16.63-20.85
		US-GC	71	17.83	0.884	15.54-19.65
		US-SD	38	17.35	0.969	14.88-19.12
	16	CMP	12	17.68	2.094	15.08-22.11
		US-CP	44	14.17	1.076	12.23-16.45
		US-GC	71	13.43	0.884	11.14-15.25
		US-SD	38	12.93	0.973	10.48-14.72
	18	US-CP	44	16.37	1.077	14.43-18.65
		US-GC	71	15.63	0.884	13.34-17.45
		US-SD	38	15.15	0.969	12.68-16.92
		CD	4	19.83	2.18	18.40-23.01

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
Pulse Duration (milliseconds)	23	CMP-SW	23	24.62	2.485	19.59-29.76
		US-CP	41	27.16	5.454	11.30-35.72
		US-GC	63	26.36	4.958	13.12-36.15
		US-SD	32	30.66	5.312	11.82-39.76
	20	BAL	8	50.08	10.438	38.26-66.48
		US-CP	41	33.99	5.453	18.13-42.54
		US-GC	63	33.19	4.958	19.95-42.97
		US-SD	32	37.48	5.311	18.65-46.58
	16	CMP	12	37.17	3.618	30.77-42.76
		US-CP	41	43.09	5.454	27.23-51.65
		US-GC	63	42.29	4.958	29.05-52.08
		US-SD	32	46.59	5.313	27.75-55.69
	18	US-CP	41	38.54	5.453	22.68-47.09
		US-GC	63	37.74	4.958	24.50-47.52
		US-SD	32	42.04	5.312	23.20-51.14
		CD	4	34.27	1.84	31.76-36.17

Table C-1. Continued

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
Inter-Pulse Interval (milliseconds)	23	CMP-SW	23	11.57	2.202	7.81-15.67
		US-CP	42	15.51	4.287	7.73-25.91
		US-GC	63	17.33	5.129	8.70-32.75
		US-SD	31	15.90	3.683	9.53-24.57
	20	BAL	8	42.08	7.278	33.53-53.70
		US-CP	42	21.65	4.287	13.86-32.04
		US-GC	63	23.46	5.129	14.83-38.88
		US-SD	31	22.04	3.683	15.66-30.71
	16	CMP	12	20.13	3.871	14.48-25.81
		US-CP	42	29.83	4.287	22.04-40.22
		US-GC	63	31.65	5.129	23.01-47.06
		US-SD	31	30.22	3.683	23.84-38.89
	18	US-CP	42	25.74	4.287	17.95-36.13
		US-GC	63	27.55	5.129	18.92-42.97
		US-SD	31	26.13	3.683	19.75-34.80
		CD	4	18.46	2.07	16.71-21.12

Trait	Temperature (°C)	Populations	N	Grand Mean	Standard Deviation	Range
Sub-Pulse Period (milliseconds)	23	CMP-SW	23	4.21	0.413	3.26-4.70
		US-CP	42	4.75	0.664	2.53-6.47
		US-GC	62	4.62	0.491	3.22-6.16
		US-SD	29	4.31	0.442	3.36-5.19
	20	BAL	8	5.18	0.264	4.80-5.66
		US-CP	42	5.25	0.664	3.03-6.97
		US-GC	62	5.12	0.492	3.72-6.66
		US-SD	29	4.81	0.442	3.85-5.69
	16	CMP	12	5.50	0.427	4.84-6.25
		US-CP	42	5.91	0.664	3.69-7.63
		US-GC	62	5.79	0.492	4.38-7.32
		US-SD	29	5.48	0.442	4.52-6.35
	18	US-CP	42	5.58	0.664	3.36-7.30
		US-GC	62	5.45	0.491	4.05-6.99
		US-SD	29	5.14	0.442	4.19-6.02
		CD	4	5.41	0.530	4.77-5.85

Table C-1. Continued

Trait	Populations	N	Grand Mean	Standard Deviation	Range
Percentage Pulse Rise-time (%)	CMP-SW	23	67.26	4.586	59.09-75.61
	BAL	8	57.52	6.466	47.19-64.52
	CMP	12	73.09	5.454	65.40-82.17
	US-CP	45	67.82	5.664	49.73-78.86
	US-GC	65	65.12	4.853	54.42-78.24
	US-SD	28	69.79	5.929	57.20-78.11
	CD	4	71.94	2.70	69.93-75.92
Call Duty Cycle (%)	CMP-SW	22	31.29	6.59	21.81-47.75
	BAL	8	47.15	9.08	32.76-58.40
	CMP	11	34.24	5.46	25.78-43.40
	US-CP	40	34.42	6.76	22.96-46.27
	US-GC	62	27.55	5.63	16.88-40.46
	US-SD	29	33.96	5.29	24.03-50.07
	CD	2	32.77	9.19	26.28-39.27

APPENDIX D: AFLP PROTOCOL

Preselective Primers:
ECO-A
MSE-A
MSE-C

Selective Primers:
A: ECO-ACT (Pet) & MSE-ATA
B: ECO-ACT (Pet) & MSE-ACA
C: ECO-ACT (Pet) & MSE-CAC
D: ECO-ACT (Pet) & MSE-CAT
E: ECO-ACT (Pet) & MSE-CAA
F: ECO-AAC (6Fam) & MSE-CAA
G: ECO-AAC (6Fam) & MSE-ACA –did not use, not good
H: ECO-AAC (6Fam) & MSE-ATA –did not use, not good

alternative : ECO-AGC & MSE-CAC –did not use, did not try

DIGLIG

Enzyme Mix for 100 samples:

T4 Ligase BUFFER	10ul
0.5M NaCl	10ul
BSA (1mg/ml)	5ul
ECOR1	25ul
MSE	10ul
T4 Ligase	20ul
H_20	20ul
Total	100ul

Reaction Mix for 100 samples:

T4 Ligase BUFFER	100ul
0.5M NaCl	100ul
BSA (1mg/ml)	50ul
MSE Adaptor (50uM)	100ul
ECO Adaptor (5uM)	100ul
Enzyme Mix	100ul
Total	550ul

Reaction Mix	5.5ul
Dilution	5.5ul
Total	11ul

Mix and centrifuge for 10 seconds
Run on thermocycler 2 hours @ 37C
Dilute with 89ul IDTE

Pre-Selective PCR

PRESEL

H_20	13.8ul
Taq BUFFER	2.0ul
dNTPs (10mM)	0.5ul
MSE-P	0.5ul
ECO-P	0.5ul
TAQ	0.2ul
Total	17.5ul

Master Mix	17.5ul
DigLig	2.5ul
Total	20.0ul

Run on program AFLPPRE

Lid 105°
Wait Auto
1 72° 2:00
2 94° 0:30
3 56° 0:30
4 72° 2:00
5 Goto 2 Rep 29
6 60° 10:00
7 Hold 4°
End

Run 10 ul on gel
Dilute rest with 40 ul IDTE

Selective PCR
-differs from Wolf protocol (Vos 1995) : doubled all reagents and added BSA

SELPCR

H_20	13.62ul
Taq BUFFER	2.0ul
dNTPs (10mM)	0.5ul
BSA (1mg/ml)	0.2ul
MSE-SEL	0.4ul
ECO-SEL (labeled)	0.08ul
TAQ	0.2ul
Total	17.00ul

Master Mix	17.0ul
PreSel	3.0ul
Total	20.0ul

AFLP SEL
Control block
Lid 105°
Wait Auto

1	94°	2:00
2	94°	0:30
3	65°	0:30
	-0.7° + 0:00	
	R 1.0 / s 0.0° /s	
	G 0.0°	
4	72.0	2:00
5	Goto 2 Rep 12	
6	94°	0:30
7	56°	0:30
8	72°	2:00
9	Goto 6 Rep 23	
10	60°	30:00
11	Hold 4°	

APPENDIX E: EXAMPLE ELECTROPHEROGRAMS OF AFLP PROFILES

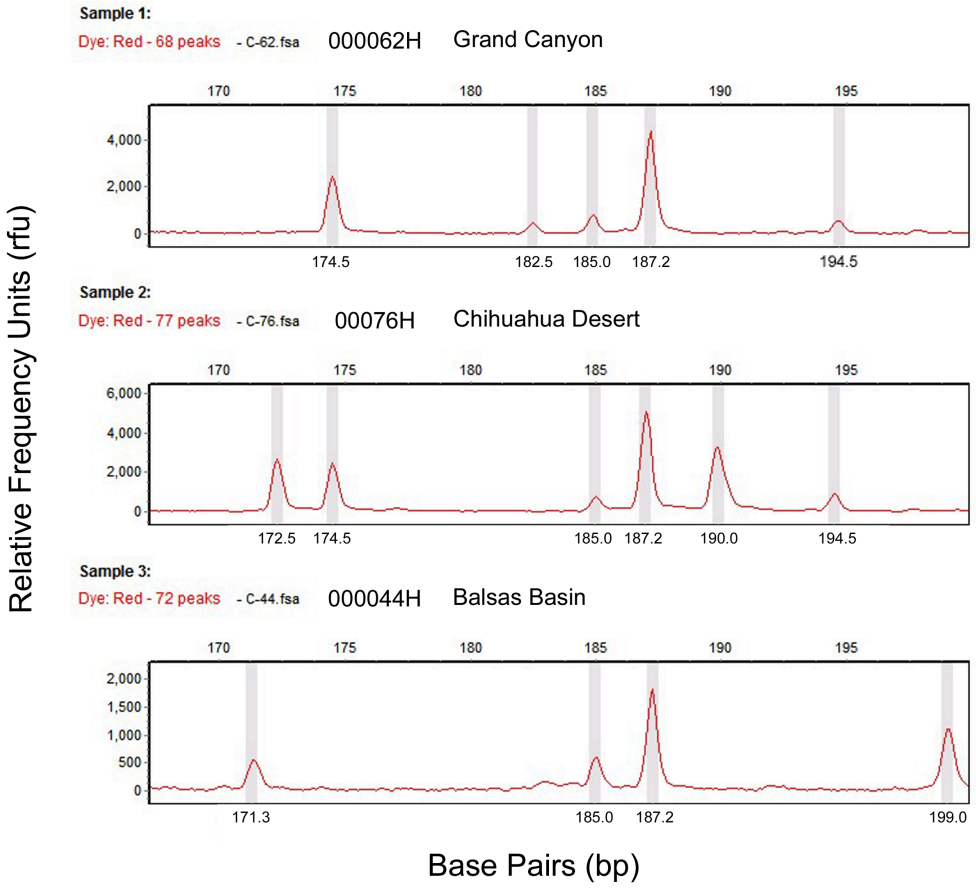

Figure E-1. Electropherogram AFLP profiles for three individuals.
The x-axis is the base pair size of each band, and the y-axis measures the intensity of each peak in relative frequency units.

APPENDIX F: AFLP DATA MATRIX

#NEXUS
[MacClade 4.06 registered to Biological Sciences , University of Missouri]
BEGIN DATA;
DIMENSIONS NTAX=101 NCHAR=727;
FORMAT DATATYPE= STANDARD MISSING=? GAP=- ;
Matrix

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VITA

Katy E. Klymus was born on May 2, 1978 and grew up in Plano, Texas. She graduated from Plano East Senior High School in 1996. She then went on to attend the University of Texas at Austin. In 2000 she earned a Bachelor of Science degree in Zoology. After graduating, Katy entered the Peace Corps and served as an Environmental Awareness volunteer in the west African nation of Benin for two and a half years. Upon returning to the USA, Katy interned in the Conservation Program at the Brookfield Zoo, in 2003. She later worked as a technician in their experimental population genetics mouse colony, as well as a seasonal zookeeper at the Children's zoo. In 2004, she returned to Austin, Texas to work as an animal care technician at the University's Túngara frog colony. Katy then moved to Columbia, Missouri to begin graduate studies at the University of Missouri in 2005. Under the guidance of Dr. Carl Gerhardt she spent most of her springs in the field, studying canyon treefrog mating behavior in the Grand Canyon, Arizona and Zion National Park, Utah. During the fall semester, Katy returned to the University for classes and conducted the molecular genetics component of her research. During her studies, Katy also got to travel to Mexico in search of her study species. In 2011, Katy earned her Doctor of Philosophy in Biological Sciences with an emphasis in Ecology and Evolutionary Biology.