

Geographic Variation in *Hyla wrightorum*: Advertisement Calls, Allozymes, mtDNA, and Morphology

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We studied geographic variation in allozymes (22 loci), mitochondrial cytochrome *b* gene sequences (575 bp), advertisement calls (pulse rate, call duration, and dominant frequency), and snout–vent length among populations of *Hyla wrightorum* and *Hyla eximia* in the United States and Mexico. Calls were only available for *H. wrightorum*, and although populations varied in some advertisement call variables, there was no indication of species level differentiation. Allozyme variation was exhibited among the *H. wrightorum* populations, but no fixed differences were discovered, and the amount of genetic divergence among populations was small ($D_m \leq 0.0643$). Seven mtDNA haplotypes were discovered among the *H. wrightorum* individuals included in this study. A single haplotype (G) was present in the Huachuca Mountains and was found only in this population restricted to southeast Arizona. Neither the Mogollon Rim nor the Sonora populations were exclusive, with some haplotypes in each being more closely related to haplotypes in the other population. Molecular data (allozymes and mtDNA), as well as the advertisement calls, support continued recognition of two species: *H. eximia* in central-southern Mexico and *H. wrightorum*, which consists of disjunct populations in the Sierra Madre Occidental of northern Mexico, the Huachuca Mountains of southeastern Arizona, and the mountains of central Arizona and western New Mexico.

THE Arizona Treefrog, *Hyla wrightorum*, has received little attention (Renaud, 1977; Sullivan, 1986) and, until recently, was often synonymized under *Hyla eximia* (Duellman, 2001). *Hyla eximia* was described by Baird (1854) with a type locality from “Valley of Mexico” (Distrito Federal), Mexico, and it occurs throughout the southern parts of the Mexican Plateau (= Mesa Central), the Sierra Madre Oriental and Sierra Madre Occidental, and the Cordillera Volcánica in central Mexico (Duellman, 2001). *Hyla wrightorum* was diagnosed by Taylor (1938) as a species separate from *H. eximia* based on the presence in the former of larger size, anterior edge of tibia with heavy brown spots and lacking a white line, and proportionately longer legs. Populations of *H. wrightorum* occur along the Mogollon Rim of central Arizona into western New Mexico, the Huachuca Mountains and adjacent Canelo Hills of southeastern Arizona, and the northern Sierra Madre Occidental of Mexico.

Following Taylor’s (1938) description, Schmidt (1953) arbitrarily listed *H. wrightorum* as a subspecies of *H. eximia*. Shortly thereafter, Blair (1960) provided evidence from mating calls indicating subspecies designation was premature; he described “Fast *eximia*” and “Slow *eximia*” populations whose pulse rates of advertisement calls at similar recording temperatures were dramatically different between southern Mexico and Arizona, and suggested the exist-

tence of two species. Jameson et al. (1966) recognized *H. wrightorum* as a subspecies of *Pseudacris regilla* based on a multivariate discriminant function analysis of 10 morphological measurements. Maxson and Wilson (1974) compared serum albumins of *H. eximia*, *P. regilla*, and *H. wrightorum* and argued that *H. eximia* and *H. wrightorum* are closely related but relatively divergent from *P. regilla*, contrary to Jameson et al. (1966). Renaud (1977) subsequently compared morphometric, allozyme, and advertisement call variation of Mogollon Rim and mainland Mexico populations and concluded that Arizona *H. wrightorum* could be distinguished from *H. eximia* of southern Mexico based on differences in size (snout–vent length, or SVL), shape, and dominant frequency of male advertisement calls.

Renaud (1977) did not find a statistically significant relationship between advertisement call dominant frequency (DF) and pulse rate (PR) against wet and dry-bulb air temperatures, water temperature, or SVL; hence no correction factors were applied in analyses of call data. However, Sullivan (1986) found a significant difference in mean pulse rate of treefrogs he recorded at Baker Lake, Arizona, leading him to conclude that pulse rate may be influenced by body temperature, contrary to Renaud’s (1977) assertion. Additionally, Sullivan (1986) found a statistically significant relationship between DF and SVL, contrary to Renaud (1977). Regard-

less of these issues, the taxonomic conclusions of Renaud (1977) were not widely accepted (e.g., Tanner, 1989). Recently, Duellman (2001) reversed his earlier position (Duellman, 1970) and recognized *H. wrightorum* and *H. eximia* as distinct species basing his conclusions in part on the call analysis of Sullivan (1986) and preliminary molecular results provided by us (EWAG and TWR).

Populations of *H. wrightorum* isolated in the Huachuca Mountains and adjacent Canelo Hills of southern Arizona have received scant attention. Knowledge of genetic and phyletic diversity is often antecedent to formulation of a conservation strategy or comparative evolutionary studies (Vane-Wright, 1996; Vogler and DeSalle, 1994). Viability of populations of *H. wrightorum* in the Huachuca Mountains is of special concern because of their geographically restricted nature (P. A. Holm and C. H. Lowe, Arizona Game and Fish Dept. Tech. Report, Phoenix, AZ, 1995, unpubl.). Small populations have the potential for low levels of genetic heterozygosity and increased inbreeding depression, which may be harmful to the population, and small populations are also more susceptible to local extinction from unpredictable changes in the environment.

Because of difficulties with previous taxonomic analyses of *H. eximia* and *H. wrightorum*, the absence of information on populations of *H. wrightorum* from southern Arizona, and the advent of modern molecular methods for assessing species status, a reevaluation of the systematics of *H. eximia* and *H. wrightorum* was warranted. We analyzed geographic variation within *H. wrightorum* by comparing advertisement call and body size, allozymes, and mitochondrial cytochrome *b* sequences among populations from the Mogollon Rim and Huachuca Mountains of Arizona and the Sierra Madre Occidental of Mexico, as well as between *H. eximia* and *H. wrightorum*.

MATERIALS AND METHODS

Collections and samples.—Male advertisement calls were recorded in the field using a Marantz PMD 430 Stereo Recorder and Sennheiser ME 80 microphone. When possible, a minimum of five calls was recorded from each individual. Snout–vent length (SVL) of each recorded individual was measured to the nearest millimeter with a hand rule, and cloacal temperatures were measured with a Weber Quick Reading Thermometer immediately after recording each frog. Frogs were recorded from several areas representative of the geographic regions em-

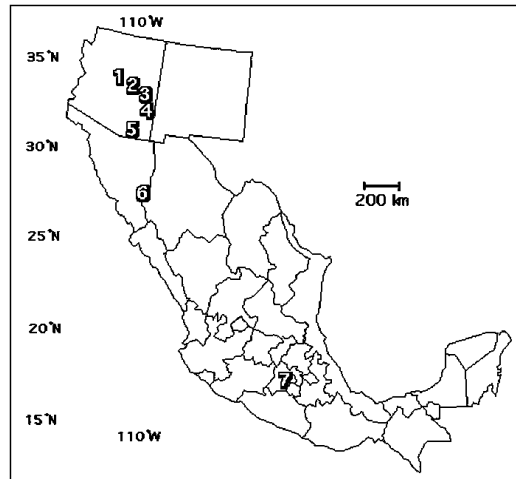


Fig. 1. Collecting localities for Arizona Treefrogs (*Hyla wrightorum*; 1–6) in Arizona, and northern Mexico, and Mountain Treefrogs (*Hyla eximia*; 7) in southern Mexico. 1 = Flagstaff; 2 = East Clear Creek; 3 = McNary; 4 = Hannagan Meadow; 5 = Huachuca/Canelo Hills; 6 = Sonora; 7 = Mexico, D.F.

phasized in this study including the Mogollon Rim, Huachuca Mountains, and Sierra Madre Occidental at Yecora (Fig. 1).

Adults were collected and tissues taken from throughout the distribution of *H. wrightorum* (Fig. 1, Materials Examined). General locations in Arizona included: Flagstaff ($n = 16$), East Clear Creek at Jones Crossing ($n = 20$), McNary ($n = 13$), 7.0 miles north of Hannagan Meadow ($n = 7$), Turkey Creek at Canelo Hills ($n = 6$), tributary to Scotia Canyon in the Huachuca Mountains ($n = 4$). Samples were also obtained from the Sierra Madre Occidental (Yecora, Mexico; $n = 10$). Liver, small intestine, and skeletal and cardiac muscle were extracted and stored in liquid nitrogen in the field, and transferred to a -70 C freezer for long-term storage. Sample size was often limited by the number of males observed calling; relatively small samples were taken from those populations with apparently small population densities. For comparison (allozymes and mtDNA), tissues representing three *H. eximia* individuals (Mexico D.F.) were obtained, as well as samples for five out-group hylid species for the mitochondrial phylogenetic analysis (see Materials Examined).

Starch gel electrophoresis procedures and analysis.—Liver and skeletal muscle were homogenized separately in a 1:1 (v:v) mixture of tissue and 0.01 M Tris-0.001 M EDTA-0.001 M mercaptoethanol, pH 6.8. Homogenates were centrifuged at 12,000 g for 10 min. at 5 C. Within 72

h, the supernatant fractions were run on horizontal starch gels between approximately 0 and 5 C. Generally, supernatant fractions were used once and refrozen at -70 C in case further analysis was necessary. Standard horizontal starch gel electrophoresis procedures were used (Murphy et al., 1996). Gels were composed of 12% hydrolyzed potato starch from Starch Art Corporation (Smithville, TX). Attempts were made to resolve 28 enzyme systems using stains of Murphy et al. (1996). Locus homologies were estimated by relative staining intensities and mobilities in specific tissues. Enzymes, loci, tissue sources, and electrophoretic conditions are listed in Appendix 1. Electromorphs were labeled a, b, c, etc., in order of decreasing anodal mobility. Allele frequencies were calculated, and Nei's (1978) unbiased genetic distances among sampled populations were calculated using Tools for Population Genetic Analysis (vers. 1.3, M. P. Miller, 1997, unpubl.).

MtDNA sequencing and phylogenetic analyses.—Mitochondrial DNA sequence data were collected from a subset ($n = 30$) of the *H. wrightorum* and *H. eximia* individuals obtained for the allozyme analysis (see Collections and Samples), as well as one individual for each of the five hylid outgroup species (*Hyla arenicolor*, *Hyla cinerea*, *Hyla squirella*, *Pseudacris cadaverina*, and *P. regilla*). DNA was isolated from small amounts of liver (~ 100 mg) following the phenol/chloroform/isoamyl alcohol protocol of Hillis et al. (1996). The polymerase chain reaction (PCR) was used to amplify a 575 bp fragment of the mitochondrial cytochrome *b* gene. Primers used to amplify this fragment were those of Ptacek et al. (1994). Approximately 50–100 ng of total DNA was used as template in a standard double-stranded PCR amplification. PCR cycle parameters for this fragment were 94 C for 30 sec, 50 C for 30 sec, and 72 C for 30 sec (40 cycles). Prior to sequencing PCR products, unincorporated nucleotides and primers were removed using Wizard PCR PrepsTM (Promega, Inc.). Double-stranded DNA templates were sequenced using a dye-labeled dideoxy terminator cycle sequencing kit (Applied Biosystems, Inc.) and an ABI 377 automated DNA sequencer (Applied Biosystems, Inc.). Sequences were analyzed and edited using the computer software program SequencherTM. The DNA sequences were aligned with Clustal W (Thompson et al., 1994). Because this fragment codes for a protein product, alignment was straightforward and unambiguous. Phylogenetic analysis of all the DNA sequences was conducted using maximum likelihood (ML), as implemented in PAUP* (vers.

4.0b10, D. L. Swofford, Sinauer Assoc., Sunderland, MA, 1999, unpubl.).

The preferred ML phylogeny was estimated following a successive approach similar to that described by Swofford et al. (1996) and Wilgenbusch and de Queiroz (2000), except many more models (56 in all) were evaluated and tested using ModelTest 3.0 (Posada and Crandall, 1998). The best model (and estimated parameters) was then used in a ML heuristic tree search (TBR branch swapping; 20 random taxon addition replicates). Also, as an alternate means of depicting haplotype relationships within *H. wrightorum*, a haplotype network was inferred by statistical parsimony, as implemented in TCS v1.13 (Clement et al., 2000).

Confidence in the ML inferred clades was assessed using both Bayesian and bootstrap methods. Bayesian analyses were performed using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001), using the general model previously identified using ModelTest. Bayesian analyses were conducted with random starting trees (as well as uniform priors) and run for 1.0×10^6 generations (four heated chains; default heating values), sampling the Markov chains at intervals of 100 generations. To determine whether the Bayesian analyses had reached stationarity, likelihoods of sample points were plotted against generation time. Sample points generated before reaching stationarity were discarded as “burn-in” samples. To ensure the Bayesian analyses were not trapped on local optima, analyses were performed twice and apparent stationarity levels were compared for convergence (Huelsenbeck and Bollback, 2001). In all analyses, the likelihood values stabilized by 2.0×10^5 generations, with the last 8000 sampled trees being used to estimate the Bayesian posterior probabilities (= proportion of trees recovering any particular clade).

Unlike nonparametric bootstrap values, which are known to be conservative estimates of clade confidence (Hillis and Bull, 1993), recent simulation studies (e.g., Wilcox et al., 2002; Alfaro et al., 2003; Erixon et al., 2003) have demonstrated that Bayesian posterior probabilities are less biased estimators of confidence and, thus, generally represent much closer estimates of true clade probabilities (referred to as “*Pc*” throughout). Also, although the Bayesian approach may be more sensitive to signal in the sequence data (i.e., provide higher confidence for short internodes; Alfaro et al., 2003), there is also an increased chance of the Bayesian method assigning higher confidence to incorrectly inferred short internodes because of the stochastic nature of the underlying model of

evolution (Alfaro et al., 2003; Erixon et al., 2003). Given this, clades with $P_c \geq 0.95$ were generally considered strongly (significantly) supported but with the caveat that relatively higher posterior probabilities for short internodes (particularly those receiving low bootstrap values) may be overestimates of confidence. For comparison to the Bayesian posterior probabilities, maximum likelihood nonparametric bootstrap values (Felsenstein, 1985) were estimated from 200 pseudoreplicates (two random sequence additions/pseudoreplicate; TBR branch swapping; implemented in PAUP*), using the best model of sequence evolution (see above).

Based on phylogenetic information from previous studies (Cocroft, 1994; Da Silva, 1997; Hedges, 1986), five outgroup hylid species (i.e., *Hyla arenicolor*, *Hyla cinerea*, *Hyla squirella*, *Pseudacris cadaverina*, and *P. regilla*) were chosen for this study. Although these studies are suggestive regarding which North American hylids may be closely related to *H. eximia* and *H. wrightorum*, the higher-level relationships among hylines is poorly understood, with the Holarctic hylines likely not representing a clade and being nested within a larger and more diverse Middle American clade (J. J. Wiens and T. W. Reeder, unpubl. data). Thus, we made no assumptions regarding which of the chosen outgroups was most closely related to *H. eximia* and *H. wrightorum* and simultaneously analyzed all taxa and rooted the phylogeny between *H. eximia* + *H. wrightorum* and the remaining taxa. However, such a rooting strategy does make the assumption that *H. eximia* and *H. wrightorum* are sister taxa (with respect to the other included species), an assumption that can be rejected if it is not possible to root the resulting phylogeny in a way that supports these two species as a clade to the exclusion of the other included outgroups.

Call analysis.—In the laboratory, analog recordings of advertisement calls were digitized at a capture rate of 22,000 datapoints per second on a Macintosh PowerPC using Canary software (vers. 1.2.1). Call duration (CD) and pulse rate (PR) were measured in the waveform mode; CD was determined by measuring the total length of a call to the nearest 0.01 sec, and PR was calculated by dividing the total number of pulses per call by the call duration. Dominant frequency (DF) of advertisement calls was obtained from each call using the spectrum mode of Canary with the following settings: frame length 16384 pts, time 2048 pts (87.5% overlap), FFT size 16384 pts, hamming filter, and

amplitude quadratic to maximize frequency precision. Peak frequency was recorded as DF for every advertisement call. An average value for each call variable was obtained from no fewer than three calls per individual, and these summary data were used for further analyses of call variation among Mogollon Rim, Huachuca Mountains, and Sonora populations.

Because call variables typically vary significantly with body size or temperature, we first assessed the relationship of call variables to male SVL and to temperature. Variables significantly influenced by temperature were adjusted with the appropriate regression equation for analysis of size-related variation. To assess similarity among allopatric populations, ANOVA was used with Tukey pairwise comparisons (Sokal and Rolf, 1981) using SYSTAT 5.2 for the Macintosh (Evanston, IL, 1992).

RESULTS

Allozymes.—Twenty-one loci were resolved (Table 1) for all populations except Mexico D.F., for which Ak, Ck, Fba, and Pk were not resolved because only liver tissue was available for analysis. Nine loci were monomorphic for all populations assayed (Aat-2, Ak, Ck, Ddh, Fba, Fumh, Iddh, Mdh-2, Pk; Table 1). Although sample size was limited ($n = 3$), the Mexico D.F. population (representing *H. eximia*) was fixed for unique allozyme alleles at three loci (Acon-2, Est-2, Sod), was polymorphic for unique alleles at two loci (Aat-1 and Gpi), and was fixed for an allele shared with at least some other populations of *H. wrightorum* at four loci (Acon-1, Idh-2, Mpi, and Pgm). The Sonora population exhibited unique allozymes at three polymorphic loci (Acon-1, Gpi, and Idh-2) and was fixed for an electromorph shared with at least some other populations of *H. wrightorum* at two loci (Idh-1 and Pgm). The Canelo and Huachuca populations shared identical character states and did not differ from Mogollon Rim populations in character state distributions except at the Mpi locus (Mpi^b for Huachuca and Canelo populations, Mpi^{ab} or Mpi^{abc} for Mogollon Rim populations). Nei's unbiased genetic distance (D_m) ranged from 0.0020 (Flagstaff vs East Clear Creek) to 0.6061 (Sonora vs Mexico D.F.; Table 2). All populations of *H. wrightorum* exhibited a D_m of at least 0.4651 from the Mexico D.F. population of *H. eximia*, but none of the populations of *H. wrightorum* were separated by D_m greater than 0.0643.

mtDNA.—Uncorrected estimates of sequence divergence among treefrog samples ranged from

TABLE 1. DISTRIBUTION OF GENOTYPES AT 21 LOCI FOR SAMPLED POPULATIONS OF *Hyla wrightorum* AND *Hyla eximia*. The alleles and their relative mobilities are designated by letters. Sample sizes are indicated below each locality. Huachuca includes Canelo Hills. ND = no data.

Locus	Flagstaff (n = 16)	East Clear Creek (n = 20)	McNary (n = 13)	Hammagan Meadow (n = 7)	Huachuca (n = 10)	Sonora (n = 10)	Mexico, D.F. (n = 3)
Aat-1	c	c	c	c	c	c	a (0.1667) b (0.8333)
Aat-2 (cath)	a	a	a	a	a	a	a
Acon-1	b (0.5000) c (0.5000)	b (0.4500) c (0.4500)	b (0.4615) c (0.5385)	b (0.4286) c (0.5714)	b (0.6500) c (0.3500)	a (0.1500) b (0.1000) c (0.7500)	a b c
Acon-2	a	a	a	a	a	a	b
Ak	a	a	a	a	a	a	ND
Ck	a	a	a	a	a	a	ND
Ddh	a	a	a	a	a	a	a
Est-2	b	b	b	b	b	b	a
Fba	a	a	a	a	a	a	ND
Fumh	a	a	a	a	a	a	a
G3pdh	a (0.4333) b (0.5667)	a (0.4000) b (0.6000)	a (0.4615) b (0.5385)	a (0.7857) b (0.2143)	a (0.4500) b (0.5500)	a (0.6000) b (0.4000)	a (0.6667) b (0.3333)
Gpi	c	c (0.8500) d (0.1500)	c (0.4615) d (0.5385)	c (0.5000) d (0.5000)	c (0.7000) d (0.3000)	b (0.2500) c (0.5500) d (0.2000)	a (0.6667) c (0.3333)
Iddh	a	a	a	a	a	a	a
Idh-1	a (0.9375) b (0.0625)	a (0.9000) b (0.1000)	a (0.9231) b (0.0769)	a (0.7143) b (0.2857)	a	a	a
Idh-2	b	b	b	b	b	a (0.1000) b (0.9000)	b
Mdh-1	a (0.2813) b (0.7188)	a (0.5500) b (0.4500)	a (0.4231) b (0.5769)	a (0.2143) b (0.7857)	a (0.4500) b (0.5500)	a (0.5000) b (0.5000)	b
Mpi	a (0.3125) b (0.3438) c (0.3438)	a (0.3250) b (0.4250) c (0.2500)	a (0.3846) b (0.5769) c (0.0385)	a (0.1429) b (0.8571)	b	a (0.1000) b (0.8000) c (0.1000)	c
Pgm	a (0.1250) b (0.7813) c (0.0938)	a (0.0750) b (0.8750) c (0.0500)	a (0.2308) b (0.7692)	a (0.0714) b (0.9286)	a (0.3500) b (0.6500)	b	c
Pk	a	a	a	a	a	a	ND
Sod	a	a	a	a	a	a	b

TABLE 2. MATRIX OF NEI'S (1978) UNBIASED D_m (ABOVE DIAGONAL) AND IDENTITIES (BELOW DIAGONAL) OF *Hyla wrightorum* AND *Hyla eximia* POPULATIONS. Population codes are as follows: 1 = East Clear Creek, 2 = Flagstaff, 3 = McNary, 4 = Hannagan Meadow, 5 = Huachucas/Canelo Hills, 6 = Sonora, 7 = Mexico D.F.

	1	2	3	4	5	6	7
1	—	0.0020	0.0072	0.0240	0.0165	0.0592	0.5067
2	0.9980	—	0.0165	0.0287	0.0221	0.0704	0.4651
3	0.9928	0.9837	—	0.0074	0.0085	0.0583	0.5237
4	0.9763	0.9717	0.9926	—	0.0154	0.0571	0.5274
5	0.8836	0.9781	0.9916	0.9848	—	0.0643	0.5590
6	0.9425	0.9320	0.9434	0.9445	0.9377	—	0.6061
7	0.6025	0.6281	0.5923	0.5901	0.5718	0.5455	—

0.0–7.6% with the greatest levels of divergences between the Mexico D.F. samples (= *H. eximia*) and the remaining individuals (= *H. wrightorum*). A total of nine unique haplotypes were discovered (Fig. 2A) within these two species. Two haplotypes, which differed by only a single nucleotide substitution, were found among the three Mexico D.F. individuals. These two haplotypes differed from all the haplotypes of *H. wrightorum* by at least 35 substitutions. Of the seven haplotypes within *H. wrightorum* (Fig. 2A–B), the most frequent was haplotype A (observed in most Mogollon Rim individuals and one Sonora individual), with haplotype B (single Hannagan Meadow individual) being derived from haplotype A. The Sonora individuals exhibited three different haplotypes (A, C, and F), and the Huachuca Mountains and Canelo Hills samples all exhibited haplotype G. The single optimal ML phylogeny inferred for the haplotypes is shown in Figure 2A (haplotype relationships identical in maximum parsimony). Within *H. wrightorum*, three different haplotype clusters are evident (i.e., G, D + E + F, and A + B + C), being separated by 5–6 substitutions (only 1–2 substitutions within clusters; Fig. 2B). The interrelationships between these haplotype clusters is not strongly supported ($P_c \leq 0.95$), with haplotype G of southeast Arizona being placed as sister to the remaining *H. wrightorum* haplotypes found on the Mogollon Rim and in Sonora. Although most of the interrelationships between haplotypes of *H. wrightorum* are weakly supported (based on Bayesian and bootstrap analyses), all the substitutions between the haplotypes are unique (i.e., no convergent transformations). And finally, neither the Mogollon Rim nor the Sonora population were exclusive, with each population possessing some individuals exhibiting haplotypes that are more closely related (or even identical) to haplotypes in the other population (e.g., haplotypes D and E of Hannagan Meadow more closely related to haplotype F of Sonora).

Advertisement calls.—For all populations as a group, temperature significantly influenced PR ($R^2 = 0.559$, intercept = 7.10, slope = 5.023, $F_{1,50} = 63.35$, $P < 0.000$; Fig. 3) and CD ($R^2 = 0.478$, intercept = 0.415, slope = -0.011 , $F_{1,50} = 45.71$, $P < 0.000$). Regression equations were also calculated separately for each population and no significant relationship was found between CD and temperature; this was because of a narrow range of temperature variation among recorded individuals such that none of the recorded populations exhibited slopes that differed from zero. Given small sample sizes, geographic proximity, and lack of obvious differences in call variables, samples from the Huachucas and Canelo Hills were combined for subsequent analysis.

Because ANCOVA assumes homogeneity of slopes among samples when slopes are not zero, ANCOVA was deemed unnecessary for further analysis. To test for differences in PR and CD among populations, ANOVA was performed by using the overall common regression equations to adjust PR and CD to a common temperature, 20 C; PR did not significantly vary among populations ($F_{2,49} = 2.11$, $P = 0.144$), but CD did ($F_{2,49} = 91.283$, $P < 0.001$; Table 3). Tukey multiple comparisons indicated the Huachucas/Canelo Hills populations were significantly different from both the Mogollon Rim and Sonora populations in CD, but the Mogollon Rim did not differ significantly from Sonora (Table 4).

For all populations of *H. wrightorum* as a group, SVL significantly influenced DF ($R^2 = 0.839$, intercept = 4.228, slope = -0.055 , $F_{1,50} = 261.47$, $P < 0.001$; Fig. 3). ANCOVA could not be employed because of heterogeneity of slopes; the regression equation from each population was used to adjust DF before using ANOVA to compare populations. When adjusted for size, DF significantly varied among populations ($F_{2,49} = 179.61$, $P < 0.001$; Table 3), and Tukey comparisons indicated significant differences among all three populations (Table 4).

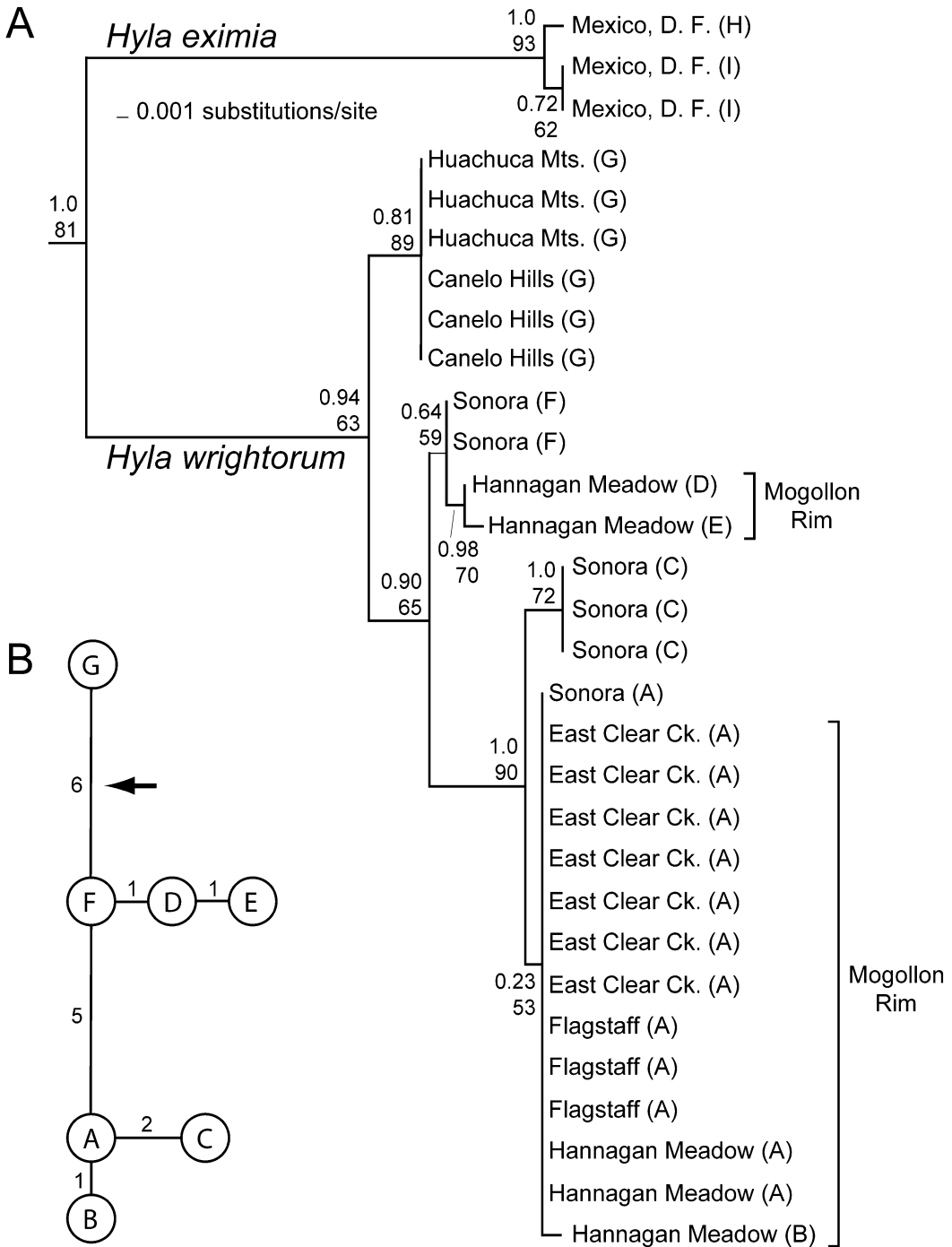


Fig. 2. (A) Single optimal maximum-likelihood phylogeny of the cytochrome *b* haplotypes within *Hyla eximia* and *Hyla wrightorum*. Letters following the individual localities denote the given haplotype. Numbers along the branches represent Bayesian posterior probabilities (above) and bootstrap percentages (below). Maximum-likelihood model (GTR + I + Γ) parameters used: Base frequencies: A = 0.28132, C = 0.31993, G = 0.10084, T = 0.29792; R-matrix: A–C = 2.34, A–G = 26.05, A–T = 2.63, C–G = 1.39, C–T = 21.20, G–T 1.00; Pinvar = 0.52; α = 0.97. (B) Haplotype network for *H. wrightorum*. Numbers along branches denote the number of nucleotide substitutions between haplotypes. The arrow denotes optimal placement of the root for this network based on the maximum-likelihood analysis.

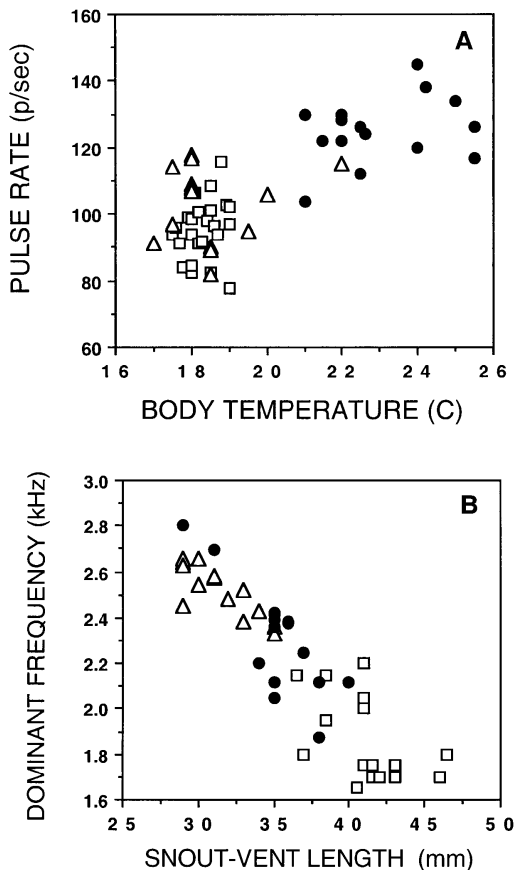


Fig. 3. Advertisement call pulse rate against body (cloacal) temperature (A) and dominant frequency against snout-vent length (B) for Central Arizona (open squares), Huachucas/Canelo Hills (solid circles), and Sonora, Mexico (open triangles) populations of *Hyla wrightorum*.

Size (SVL) also varied among populations (AN-OVA, $F_{2,49} = 74.576$, $P < 0.001$; Table 4); Tukey pairwise comparisons indicated Huachucas/Canelo Hills frogs were significantly smaller than Mogollon Rim frogs, and Sonora frogs were smaller still (Table 4).

DISCUSSION

Geographic variation and its significance.—Within *H. wrightorum*, allozyme evidence indicates low levels of differentiation among populations of the Mogollon Rim (D_m ranged from 0.0020–0.0287) and between the Huachucas and Mogollon Rim populations (maximum D_m was 0.0221). The only qualitative differences between Mogollon Rim and Huachuca Mountain populations were at the *Idh-1* and *Mpi* loci where the Huachuca population was monomor-

phic at each locus, whereas the Mogollon Rim populations were polymorphic. However, a greater level of differentiation exists between both Arizona populations (i.e., Mogollon Rim and Huachuca Mountains) and the Sonora population of *H. wrightorum* ($D_m = 0.0643$ between Huachucas and Sonora; $D_m > 0.0570$ between Mogollon Rim and Sonora). The Sonora population showed qualitative but no fixed differences between the Huachuca Mountains and Mogollon Rim with several alleles occurring only in the Sonora population (Table 1).

The Mexico D.F. population of *H. eximia* was highly divergent from all samples of *H. wrightorum* (minimum $D_m = 0.4651$) with several apparently fixed qualitative differences (Table 1). For instance, *Aat-1^c* was fixed among all populations of *H. wrightorum* (total sample size = 76), whereas *H. eximia* uniquely exhibited the *Aat-1^a* and *Aat-1^b* morphs. Similarly, all populations of *H. wrightorum* were monomorphic at the *Acon-2*, *Est-2*, and *Sod* loci, whereas *H. eximia* was fixed for alternative alleles.

Phylogenetic analysis of the mitochondrial cytochrome *b* data suggests that the haplotypes of *H. wrightorum* from the Mogollon Rim and Sonora are more closely related to each other than to haplotype G in the Huachuca Mountains and Canelo Hills (Fig. 2). Also, neither the Mogollon Rim nor the Sonora population is exclusive, with some haplotypes of one population being more closely related to haplotypes in the other population. Most (80%) of the treefrogs sampled from the Mogollon Rim exhibited haplotype A, with this common haplotype also showing up in a single Sonoran individual. In fact, this is the only instance of a shared haplotype between these major populations. All other evidence of nonexclusivity is from unique haplotypes. Two individuals from Hannagan Meadow of the Mogollon Rim each possessed unique haplotypes (D and E) that were relatively divergent from the other Mogollon Rim haplotypes (6–8 substitutions; Fig. 2B) and more closely related to the Sonoran haplotype F (Fig. 2A). A similar situation involves haplotype C of Sonora, with this haplotype being much more similar to the common haplotype A of the Mogollon Rim (differing by only two substitutions) than to Sonoran haplotype F (differing by seven substitutions; Fig. 2B). Because of the currently disjunct nature of the Mogollon Rim and Sonora populations, the nonexclusivity and sharing of haplotype A is not likely the result of recent gene flow. Instead, this pattern suggests these populations have been isolated for some time, but insufficient time has passed for random lineage sorting to result in exclusive (re-

TABLE 3. TEMPERATURE ADJUSTED (20 C) ADVERTISEMENT CALL VARIABLES (PR AND CD), SVL ADJUSTED DF, AND SVL FOR ALLOPATRIC POPULATIONS OF *Hyla wrightorum*. Values are mean \pm SE. Huachucas includes Canelo Hills.

Population	PR (p/s)	CD (s)	DF (kHz)	SVL (mm)	<i>n</i>
Central AZ	104.3 \pm 1.82	0.240 \pm 0.006	2.007 \pm 0.013	41.1 \pm 0.49	23
Huachucas	110.0 \pm 2.75	0.119 \pm 0.007	2.190 \pm 0.037	35.3 \pm 0.69	15
Sonora	110.3 \pm 3.36	0.223 \pm 0.007	2.291 \pm 0.017	31.4 \pm 0.60	14

ciprocally monophyletic) populations. The phylogenetic analysis placed haplotype G as the sister lineage to the group containing the remaining haplotypes of *H. wrightorum*; however, this basal position for haplotype G is only weakly supported ($P_c = 0.90$ for the group excluding haplotype G; Fig. 2A). In fact, this haplotype found in the Huachuca Mountains and Canelo Hills is essentially equally divergent from the haplotype D + E + F cluster as is the haplotype A + B + C cluster (Fig. 2B). Thus, these data suggest these three major populations (i.e., Mogollon Rim, Huachuca Mountains/Canelo Hills, and Sonora) may have been evolving independently from each other for approximately the same amount of time, with the apparent exclusivity and lower haplotypic diversity in the Huachuca Mountains population resulting from a lower population number in this restricted region of southeast Arizona. This is also reflected (though weakly) in the allozyme data, with similar levels of genetic divergence among populations (Table 2) and the Huachuca population showing the lowest level of heterozygosity and lowest percentage of polymorphic loci (Table 5). These genetic data may have implications for management of this isolated population of *H. wrightorum*.

The minimum number of differences between haplotypes of *H. wrightorum* and *H. eximia*

is 35 nucleotide substitutions. This far exceeds the maximum amount of divergence among haplotypes of *H. wrightorum* (i.e., 13 substitutions). The magnitude of allozymic and mtDNA sequence divergence between the Arizona + Sonora populations (= *H. wrightorum*) and the population from Mexico D.F. (= *H. eximia*) supports the relatively long evolutionary independence between these two lineages.

Comparisons of advertisement call components revealed statistically significant differences in SVL adjusted DF and temperature adjusted CD among all sampled populations of *H. wrightorum*. Frogs from the Huachucas had size-adjusted DFs nearly 200 Hz higher than those of the Mogollon Rim, and Sonoran frogs were about 100 Hz higher than those from the Huachucas. However, there were no significant differences among populations in temperature adjusted PR (Table 4). In fact, the Sonora and Huachuca Mountain samples exhibited nearly identical PRs, whereas the Mogollon Rim population was slightly lower. Pulse rate is perhaps the most important call component in mate recognition in anurans (Gerhardt, 1994; Gergus et al., 1997), yet PR has not significantly diverged among populations of *H. wrightorum* sampled in this study. Based upon PR alone, females from any particular population would probably iden-

TABLE 4. PAIRWISE PROBABILITY MATRICES FOR TUKEY MULTIPLE COMPARISONS AMONG CENTRAL ARIZONA, HUACHUCAS (INCLUDES CANELO HILLS) AND SONORA POPULATIONS OF *Hyla wrightorum*. PRs and DFs are above the diagonal of the first and second panels, respectively. CDs and SVLs are below the diagonals of the first and second panels, respectively.

	Central Arizona	Huachucas	Sonora
PR and CD			
Central Arizona	—	0.231	0.221
Huachucas	0.014	—	0.998
Sonora	0.000	0.000	—
DF and SVL			
Central Arizona	—	0.000	0.212
Huachucas	0.000	—	0.000
Sonora	0.000	0.000	—

TABLE 5. AVERAGE HETEROZYGOSITIES AND PERCENT POLYMORPHIC LOCI FOR POPULATIONS OF *Hyla wrightorum* REPRESENTED BY SAMPLES OF 10 OR MORE INDIVIDUALS. Huachucas includes Canelo Hills.

Locality	Average Heterozygosity	% Polymorphic loci
Flagstaff	0.1211	28.6
East Clear Creek	0.1325	33.3
McNary	0.1426	33.3
Huachucas	0.1105	23.8
Sonora	0.1190	28.6

tify a male from any other population of *H. wrightorum* as a potential mate.

Despite the fact that DF and CD have diverged among populations of *H. wrightorum*, these small differences are probably not biologically significant (Gerhardt, 1994). Differences in DF and CD do imply that these allopatric populations may have differentiated to a relatively minor extent. Significant differences in SVL also suggest independent evolution among these allopatric entities (Tables 4–5). These results are concordant with those of others (Taylor, 1938; Renaud, 1977; Sullivan, 1986). Both Renaud (1977) and Taylor (1938) used differences in SVL, in part, to argue for the specific recognition of *H. eximia* and *H. wrightorum*. Unfortunately, no samples are available for analysis of population variation in call parameters of *H. eximia*.

Blair (1960) analyzed calls of a small number of individuals of *H. eximia* from southern Mexico, and based upon significant differences in PR, distinguished “Fast *eximia*” from “Slow *eximia*.” Although cloacal temperatures were not reported, both air and water temperatures were. Because *H. wrightorum* usually call while partially submerged in water, water temperature probably provides a relatively close approximation of cloacal temperature; “Slow *eximia*” were recorded between 21 and 23 C and had PRs between 41 and 53 p/s, whereas “Fast *eximia*” and *H. wrightorum* from Arizona were recorded between 19 and 22 C had PRs between 100 and 136 p/s. At roughly comparable water temperatures, PRs of “Slow *eximia*” are roughly half those of “Fast *eximia*” and *H. wrightorum*. Because this magnitude of difference is common for pairs of sympatric species (Gerhardt, 1994), Blair’s (1960) results are very suggestive of cryptic species within *H. eximia* of Mexico.

Taxonomic implications.—All the available evidence (i.e., advertisement calls, allozymes and mtDNA) strongly supports the hypothesis that *H. wrightorum* (Mogollon Rim, Huachuca Mountains, and northern Sierra Madre Occidental)

and *H. eximia* (southern Sierra Madre Occidental, southern Sierra Madre Oriental, and Mesa Central) are on separate evolutionary trajectories and represent independent evolutionary species (following Frost and Hillis, 1990; see also Wiley, 1978). Although we were unable to incorporate samples of *H. eximia* from the northwestern extent of its distribution (southern Durango; those populations geographically closest to *H. wrightorum*) in our molecular analyses, the most recent distributional information for these two species suggests they are allopatric (~300 km gap; Duellman, 2001); further supporting their evolutionary independence.

With respect to the status of the major populations of *H. wrightorum* (i.e., Mogollon Rim, Huachuca Mountains, and northern Sierra Madre Occidental), each of these currently is allopatric. However, the low genetic differentiation between these populations indicates this isolation likely occurred relatively recently (i.e., late Pleistocene, 700 kya–11 kya; Van Devender, 1990; Thompson and Anderson, 2000) since genetic differentiation (allozymes and mtDNA) between these populations is relatively low. No apparently fixed allozyme differences are present between these populations. Although the effective population size of the mitochondrial genome is much smaller than that of the nuclear genome, there has still been insufficient time for lineage sorting to make the Mogollon Rim and Sierra Madre Occidental populations reciprocally monophyletic. The mitochondrial distinctiveness of the Huachuca populations is likely the result of the smaller size of this greatly restricted population, thus allowing more rapid haplotype fixation. And finally, there has been no significant divergence between these major populations in advertisement call PR. Thus, in spite of the allopatric nature of these populations, evidence of significant divergence between them is lacking, and we refrain from making any formal taxonomic recommendations based solely on allopatry as evidence of evolutionary independence.

MATERIAL EXAMINED

Museum abbreviations follow Leviton et al. (1985). The lowercase letters following the catalog numbers indicate the type of data collected from each specimen, as follows: a, allozymes; m, morphology; d, mtDNA. *Hyla eximia*: Mexico: Mexico D.F.: approximately 0.25 miles east of Rio Frio on Old road to Puebla (MVZ 149755–149757; a & d). *Hyla wrightorum*: United States: Arizona: Apache County: 1 mile east of McNary on highway 260 (LACM 141101–141114; a, m & d); Cochise County: Tributary to Scotia Canyon, 0.8 miles north of FR 78 (ASU 30915–30918; a, m & d); Coconino County: 12 miles northwest of Flagstaff on FR 222 (LACM 141085–141100; a, m & d), East Clear Creek at Jones's Crossing (LACM 141115–141137; a, m & d); Greenlee County: 7.0 miles north of Hannagan Meadow on highway 191 (ASU 30907–30914; a, m & d); Santa Cruz County: Turkey Creek at Canelo (ASU 30919–30924; a, m & d); Mexico: Sonora: 0.5 miles west of Yecora (ASU 30926–30935; a, m & d). *Hyla arenicolor*: United States: Arizona: Cochise County: Chiricahua Mountains, 0.25 miles northeast of Southwest Research Station (LSUMZ 48780, d). *Pseudacris cadaverina*: United States: California: Riverside County: San Diego State University Santa Margarita Ecological Preserve (SDNHM 69035, d). *Hyla cinerea*: United States: Louisiana: Jefferson Parish: Jean Lafitte National Historic Park (LSUMZ 48181, d). *Pseudacris regilla*: United States: California: Riverside County: San Diego State University Santa Margarita Ecological Preserve (SDSU 3963, d). *Hyla squirella*: United States: Louisiana: Jefferson Parish: Jean Lafitte National Historic Park (LSUMZ 48185, d).

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