**Phylogeography of declining relict and lowland leopard frogs in the desert Southwest of North America**

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Abstract

We investigated the phylogeography of the closely related relict leopard frog (*Rana onca*) and lowland leopard frog (*R. yavapaiensis*) – two declining anurans from the warm-desert regions of southwestern North America. We used sequence data from two mitochondrial DNA genes to assess 276 individuals representing 30 sites from across current distributions. Our analysis supports the previously determined phylogenetic break between these taxa, and we found no admixing of *R. onca* and *R. yavapaiensis* haplotypes within our extensive sampling of sites. Our assessment, however, further divided *R. yavapaiensis* into two distinct mtDNA lineages, one representing populations across Arizona and northern Mexico and the other a newly discovered population within the western Grand Canyon, Arizona. Estimates of sequence evolution indicate a possible Early Pleistocene divergence of *R. onca* and *R. yavapaiensis*, followed by a Middle Pleistocene separation of the western Grand Canyon population of *R. yavapaiensis* from the main *R. yavapaiensis* clade. Phylogeographic and demographic analyses indicate population or range expansion for *R. yavapaiensis* within its main distribution that appears to predate the latest glacial maximum. Species distribution models under current and latest glacial climatic conditions suggest that *R. onca* and *R. yavapaiensis* may not have greatly shifted ranges. Our data supports the designation of *R. onca* as a distinct taxon, and additionally points to the uniqueness of the isolated population of *R. yavapaiensis* within the western Grand Canyon.

## Introduction

The relict leopard frog, *Rana onca* (= *Lithobates onca*) and the lowland leopard frog, *R. yavapaiensis* (= *L. yavapaiensis*), occupy springs, streams, and wetlands within warm-desert regions of southwestern North America. In recent years, both of these closely related frogs have experienced population declines and broad range contractions (Clarkson & Rorabaugh 1989; Bradford, Jaeger & Jennings 2004; Sredl 2005). As an apparent regional endemic, *R. onca* has suffered the worst and is currently managed under a federally reviewed conservation agreement and strategy. Previous phylogenetic analysis based on mitochondrial DNA (mtDNA), nuclear DNA markers, and morphology revealed that these frogs were distinct taxa but at a shallow level of divergence, which led to the speculation that this level of difference "probably" represents relatively recent, Late Pleistocene-Holocene isolation (Jaeger et al. 2001). Further evidence that these taxa are closely related was subsequently provided in a broader phylogenetic analysis of ranid frogs in which a lower than species-level distinction was implied (Hillis & Wilcox 2005).

The "minimum historical range" of *R. onca* included the eastern fringe of the Mojave Desert within the drainages of the Virgin and Muddy rivers and adjacent portions of the Colorado River in the region of southwestern Utah, northwestern Arizona, and southern Nevada (Bradford et al. 2004). It now occurs naturally only at a few sites along the Colorado River in Nevada (Jaeger et al. 2001; Bradford et al. 2004). Whether *R. onca* once occurred further south on the Lower Colorado River is not clear (Bradford et al. 2004), but the Bill Williams drainage which joins the Lower Colorado River below sites occupied by *R. onca* (Fig. 2.1a) contains *R. yavapaiensis* populations (Jaeger et al. 2001). *Rana yavapaiensis* is more widespread and primarily occurs in the higher elevation

uplands of the Sonoran Desert in Arizona extending south into northern Sonora, Mexico and east into New Mexico where this frog is nearly extirpated (Platz & Frost 1984; Jennings & Hayes 1994; Jennings 1995; Sredl 2005). Populations of purported *R. yavapaiensis* from more southern reaches of the Lower Colorado River and the adjacent Imperial and Mexicali valleys of southern California and northern Baja are believed to be extinct (Vitt & Ohmart 1978; Clarkson & Rorabaugh 1989, Jennings & Hayes 1994).

Previously, Jaeger et al. (2001) had rejected the hypothesis that *R. yavapaiensis* occurred within the current range of *R. onca*, including in their analysis samples from a now extinct population on the Virgin River (site LF in Fig. 2.1a) formerly identified as containing *R. yavapaiensis* (Platz & Frost 1984). Provokingly, a recent discovery of an isolated population of related leopard frogs from a tributary to the Colorado River (Surprise Canyon; site SU in Fig. 2.1a) in the western Grand Canyon has raised further questions about the history of the *R. onca-yavapaiensis* group in that a tentative mtDNA assessment of a single sample from this newly discovered population showed that it grouped more closely with *R. yavapaiensis* (Gelczis & Drost 2004).

The Southwest deserts have complex biogeographic histories, and desert biotas show the genetic influence of major historical events, some of which implicate pre-Pleistocene vicariance (Hafner & Riddle 2005). Quaternary climatic oscillations, however, have greatly affected environmental conditions in these deserts (e.g. Betancourt et al. 1990; Thompson & Anderson 2000), and several warm-desert taxa with distributions in the regions occupied by *R. onca* and *R. yavapaiensis* display genetic structures impacted by the most recent (Late Pleistocene - Holocene) climatic changes (e.g. Riddle et al. 2000; Douglas et al. 2006; Fehlberg & Ranker 2009). For example, low mtDNA diversity in

populations of the red-spotted toad (*Bufo punctatus*) within the northeastern Sonoran Desert was interpreted as evidence of range expansion into this region following the development of warmer climatic conditions in the Middle to Late Holocene (Jaeger et al. 2005). Anurans, in general, may be especially susceptible to changes in climatic factors because they are exothermic, have permeable skins, and many lay unshelled eggs dependent on surface waters (Blaustein et al. 2001).

Both *R. onca* and *R. yavapaiensis* show affinities for warmer climatic conditions, although *R. yavapaiensis* does not generally occur in the warm lowlands of the Sonoran Desert. The stream and wetland habitats occupied by these frogs have undergone substantial changes throughout modern times (Bradford et al. 2004; Sredl 2005) and presumably dramatic changes have occurred during Quaternary climatic oscillations. These fluctuations likely caused periods when aquatic habitats were broader and better connected allowing dispersal among populations and regions, and periods of isolation when habitats were reduced and fragmented. The climatic conditions that favor these frogs, however, may be more subtle than glacial-interglacial (pluvial-interpluvial) patterns.

The purpose of our study was to gain further insight into the evolutionary history of *R. onca* and *R. yavapaiensis* in light of the recent discovery of the purported population of *R. yavapaiensis* in the western Grand Canyon. We expand on the analysis of Jaeger et al. (2001) by obtaining samples from numerous sites across the extant ranges of these species, and define lineages of mtDNA genes through phylogeographic analyses. To corroborate genetic signals, we evaluate sequence data using demographic analyses (i.e. mismatch distribution and neutrality tests). We also explore independent scenarios of

late Quaternary population histories using species distribution models (SDMs, e.g. Peterson 2001; also known as ecological niche models) and project these models onto reconstructions of climatic conditions during the latest glacial maximum (e.g. Carstens & Richards 2007; Waltari et al. 2007).

## Materials and Methods

### *Sampling*

We collected or acquired tissue samples predominantly from animals captured and released, and assessed 276 samples of our target species from 30 sites (Fig. 2.1a; Table 2.1, Table 2.2). These samples included: 51 *R. onca* from five sites in southern Nevada and one site in northwestern Arizona (the LF site in Fig. 2.1a); 202 *R. yavapaiensis* samples from 23 sites in Arizona and northern Mexico; and 23 samples from the population in Surprise Canyon, Arizona. We included an additional 36 samples from four sites in southern Sonora collected at locations thought to represent *R. yavapaiensis* sites but that revealed divergent mtDNA we interpret tentatively as representing *R. magnaocularis* (Frost & Bagnara 1976; see below). We used samples of *R. forreri* and an undescribed ranid species (*Rana* 'species 8') as outgroups based on their close phylogenetic relationship to our target taxa (Hillis & Wilcox 2005).

### *Laboratory Methods*

We isolated total genomic DNA using phenol-chloroform extraction, and assessed the entire 1035 base pairs (bp) of NADH dehydrogenase subunit 2 (ND2) for all samples.

For phylogenetic analysis we sequenced exemplars of each ND2 haplotype ($n$ = 23) for an additional 916 bp segment of cytochrome *b* (Cyt*b*). We used primers L3880 and H6033 (Riddle et al. 1993) to amplify the ND2 gene, and for sequencing replaced the reverse primer with two internal primers, H5532 (Macey et al. 2001) and H23C (designed for this study; 5`- GAAATTCCTTGA AGGACCTCAGG - 3`). To amplify and sequence Cyt*b,* we used modified primers of MVZ15-L and CytbAR-H (Vences et al. 2004).

We conducted amplifications by polymerase chain reaction at annealing temperatures between 53-57 °C using *Ex Taq* Polymerase Premix (Takara Mirus Bio, Inc., Madison, WI, USA), and purified products with ExoSAP-IT (USB Corp., Cleveland, OH, USA). We conducted fluorescence-based cycle sequencing using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1, with electrophoresis on an ABI 3130 automated sequencer (Applied Biosystems, Inc., Foster City, CA, USA). We aligned sequences using SEQUENCHER v. 4.6 (Gene Codes Corp., Inc., Ann Arbor, MI, USA), and verified alignments against those of other ranids accessed from GenBank (Lee et al. 1999; Macey et al. 2001).

*Phylogeographic Analyses*

We calculated haplotype and nucleotide diversity using ARLEQUIN v. 3.11 (Excoffier et al. 2005) and mean pairwise sequence divergences (uncorrected *p*-distances) using MEGA v. 4 (Tamura et al. 2007). Prior to phylogenetic analysis of the concatenated (ND2 + Cyt*b*) sequence data of the haplotype exemplars, we applied the partition homogeneity test (Farris et al. 1995) in PAUP* v. 4.0b10 (Swofford 2002) which indicated that the two genes were congruent ($P$ = 1.00). We assessed phylogenetic patterns using the

concatenated data under the criteria of Maximum Parsimony (MP) in PAUP* and Bayesian inference (BI) in MRBAYES v. 3.1.2 (Ronquist & Huelsenbeck 2003).

We generated unweighted MP trees employing 1000 non-parametric bootstrap replicates, heuristic search with 10 random stepwise additions, and tree-bisection-reconnection branch-swapping. To select appropriate models for BI, we used MRMODELTEST v. 2.2 (Nylander 2004) under the Akaike Information Criterion (AIC; Posada & Buckley 2004). We evaluated preliminary runs for best fit partitioning schemes using Bayes factors on the harmonic mean marginal likelihood values (Nylander et al. 2004). Final analyses were run with the Hasegawa-Kishino-Yano (HKY) model for the combined $1^{st} + 2^{nd}$ codon positions and the General Time Reversible (GTR) model for the $3^{rd}$ codon position for both genes, with equal rates of substitution between nucleotide positions.

For BI runs, we unlinked model parameters across character partitions and left the Metropolis-coupled Markov chain Monte Carlo on default, except we set the heating parameter to 0.1 in order to keep state swap frequencies between 10% and 70%. The 50% majority-rule consensus tree and associated posterior probabilities used for final interpretations were based on 3 runs of 4 million generations each. Trees were sampled every 100 generations with the first 25% of sampled trees discarded as burn-in after confirming chain stationarity using the program TRACER v. 1.4 (Rambaut & Drummond 2007).

To assess divergence times, we employed a molecular clock approach, while recognizing the potential limitations with these interpretations (e.g. Edwards & Beerli 2000; Arbogast et al. 2002). Molecular clock evaluations in anurans have often been

based on a rate estimated by Macey et al. (1998) for the separation of European and Asian bufonids. This rate of 1.38% sequence divergence between lineages per million years, or $\mu = 6.9 \times 10^{-9}$ substitutions/site/year (s/s/y), was based on partial ND1, ND2, and the intervening tRNAs, but it has been applied widely as an estimate, although probably a conservative one, for both Cyt*b* and ND2 (e.g. Jaeger et al. 2005; Austin & Zamudio 2008). This clock has been recalculated for only the ND2 gene in the genus *Eleutherodactylus* (Crawford 2003) which resulted in a mutation rate of 1.91% ($\mu = 9.57 \times 10^{-9}$ s/s/y). A much faster rate of 3.6% ($\mu = 1.8 \times 10^{-8}$ s/s/y) has been applied to Cyt*b* in European ranid species (e.g. Babik et al. 2004).

To estimate the time to the most recent common ancestor, we applied the slower and faster substitution rates in the coalescence-based program BEAST v. 1.4.8 (Drummond & Rambaut 2007). Prior to estimation, we tested the concatenated (haplotype) data set without outgroups for rate heterogeneity using a likelihood ratio test (Huelsenbeck & Crandall 1997) in PAUP*, which failed to reject the molecular clock assumption ($\chi^2 = 14.88$, d.f. = 21, $P = 0.83$). We evaluated partitioning of the concatenated sequence data using Bayes factors, and for analysis, we used a strict clock and partitioned using models HKY for the combined $1^{st} + 2^{nd}$ codon positions and GTR for the $3^{rd}$ codon position obtained from MRMODELTEST. We also assessed coalescent models of constant population size, exponential growth, expansion growth, and Bayesian skyline using Bayes factors, and selected constant population size. For final analysis, we conducted two Markov Chain Monte Carlo (MCMC) runs of 20 million generations each, sampling every 2000 generations, with the first 10% discarded as burn-in. For interpretation, we combined runs and used TRACER to examine the estimated sample sizes (ESS) to avoid

poor estimates of the parameters (ESS < 200) and to depict means and credibility intervals (CI).

*Population Analyses*

Given the expected shallow intraspecific genetic structure (Jaeger et al. 2001), we evaluated the complete ND2 data set of our taxa using a median-joining network (Bandelt et al. 1999) constructed in NETWORK v. 4.2.0.1 (www.fluxus-engineering.com). We evaluated isolation by distance among sites (pairwise $F_{st}$ -values versus Euclidean geographic distances) using a Mantel test in the program AIS (Miller 2005). We also applied a series of demographic genetic approaches to assess the ND2 data of *R. yavapaiensis*, but do not present these analyses for *R. onca* and the Surprise Canyon population as these taxa were limited in geographic scope and genetic variation (see Results).

We used mismatch distributions to test for sudden demographic expansion (Rogers & Harpending 1992; Schneider & Excoffier 1999) in *R. yavapaiensis* using ARLEQUIN, and estimated population expansion parameters $\tau$ (time since expansion expressed in units of mutational time), $\theta_0 = 2\mu N_0$, and $\theta_1 = 2\mu N_1$ (where $N_0$ and $N_1$ are the estimated number of females before and after the expansion). For sudden expansion, we approximated the beginning of the time of expansion using the formula $t = \tau/2\mu$, where t is the time measured in years since expansion and $\mu$ is the per-sequence mutation rate per generation (Rogers & Harpending 1992). We assumed ND2 rates of both $7.1 \times 10^{-6}$ and $9.9 \times 10^{-6}$ substitutions/locus/year (from above) and a two-year generation time for female *R. yavapaiensis* (Sredl et al. 1997). For comparison, we conducted neutrality tests of Fu's

$F_s$ (Fu 1997) in Arlequin and $R_2$ (Ramos-Onsins & Rozas 2002) in DNASP v. 4 (Rozas et al. 2003).

*Species Distribution Modeling*

We used the program MAXENT v. 3.3.1 (Phillips et al. 2006) to develop SDMs based on recent occurrence records and 19 bioclimatic layers representing trends, seasonality, and extremes of temperature and precipitation. We assumed in these SDMs that species distributions were determined by climate, thus ignoring potentially important features limiting frog distributions such as surface hydrology and biotic interactions (other than those driven by climate). Our emphasis, however, was on exploring broad geographic shifts in potential habitat based on changes in climate. We also made the simplifying assumption that these frogs did not shift ecological niches in response to climatic changes (niche conservatism; Wiens & Graham 2005).

We used bioclimatic data from the WorldClim database v. 1.4 with resolution of 2.5 minutes (~ 5 km; www.worldclim.org; Hijmans et al. 2005) and obtained occurrence records of *R. onca* and *R. yavapaiensis* from museum collections, literature references, and a regional database (Table 2.3). Our genetic sampling, however, revealed frogs with divergent mtDNA at four locations purported to be *R. yavapaiensis* sites in southern Sonora (Fig. 2.1a), within the Plains of Sonora and Sinaloan thornscrub biomes. Because of this taxonomic uncertainty, we excluded these four sites, as well as seven other records within the boundaries of the same lower elevation biomes within Sonora. For occurrence records that lacked coordinates or associated uncertainty, we derived estimates using the 'Georeferencing Calculator' (http://herpnet.org). We also excluded occurrence records that lacked acceptable geographic description or had an uncertainty greater than 5 km.

The final data set included 27 locations of *R. onca* within its historical distribution (Bradford et al. 2004), 270 locations of *R. yavapaiensis*, and 17 locations of purported *R. yavapaiensis* from southern California.

For MAXENT runs we used logistic regression under default settings (except for random seed) and averaged 20 replicate bootstrap models per species. We assigned 85% of occurrence records for model training and 15% for model testing. The SDMs were then projected onto simulated past climate data (Thompson & Anderson 2000) representing the latest glacial maximum (approximately 21,000 years before present) derived from two climatic models – Community Climate System Model (CCSM; Collins et al. 2006) and Model for Interdisciplinary Research on Climate (MIROC; Hasumi & Emori 2004). We explored the impact of various masks on SDMs, including generating models using masks based on appropriate ecoregions for each species. The various approaches generally converged on similar overall patterns, and we present models developed using restricted rectangular masks for *R. onca* (NW corner 38.25$^{o}$, -118.67$^{o}$; SE corner 31.46$^{o}$, -111.50$^{o}$) and *R. yavapaiensis* (NW corner 38.04 $^{o}$, -118.63$^{o}$; SE corner 25.50$^{o}$, -105.63$^{o}$). Habitat suitability was displayed as two categories in ARCGIS v.9.2. (ESRI, Inc., Redlands, CA 2007) with the lowest probability habitat defined as the lowest training presence threshold. This threshold presents suitable habitat as having values at least as high as that of all the occurrence records (Pearson et al. 2007).

## Results

### *Phylogeographic Analyses*

Our assessment of ND2 resulted in the identification of 2 *R. onca* and 21 *R. yavapaiensis* haplotypes for which we generated additional Cyt*b* data on exemplars (Table 2.1). The pairwise number of nucleotide differences among the concatenated haplotypes was at least 45 (out of 1951) between *R. onca* and *R. yavapaiensis*, with an uncorrected *p*-distance of 0.022. We identified six divergent haplotypes (based on ND2) from four locations in Sonora (Fig. 2.1a), and sequenced representative samples for Cyt*b* to include in the phylogenetic analysis. These divergent samples differed from *R. onca* and *R. yavapaiensis* by a minimum of 142 nucleotides resulting in an uncorrected *p*-distance of 0.07 to the nearest ingroup taxa (*R. onca*). We tentatively identify these samples as representing *R. magnaocularis* as our sequences were little different from that we derived for an adult specimen of *R. magnaocularis* (data not shown) collected from the Río Urique in Chihuahua (number MSB 75171, Museum of Southwestern Biology, University of New Mexico). We also sequenced three of our samples for a partial segment of mtDNA 12S and compared these with published sequences (see Pfeiler & Markow 2008) for species in the *R. berlandieri* subgroup (*Scurrilirana* clade of Hillis & Wilcox 2005). Our samples were identical (403 bp) to a sample from Sierra El Aguaje in southern Sonora (GenBank: EU728669) and closely related to a *R. magnaocularis* sample from Nayarit (GenBank: AY115131). As previously noted by Pfeiler and Markow (2008), this haplotype was not closely related to a purported *R. magnaocularis* sample from near Nuri, Sonora (GenBank: AY779239). Within the region of the Río Yaqui and Río Moctezuma, where our samples were acquired, considerable genetic variation among topminnows, genus *Poeciliopsis*, has been associated with river drainages (Quattro et al. 1996), and it is possible that leopard frogs may also demonstrate similar phylogeographic

structure. As previously suggested by Pfeiler and Markow (2008), further assessments are necessary clarifying the phylogenetic and taxonomic relationships among leopard frogs in the region.

Maximum parsimony analysis of the concatenated data set resulted a single tree (length = 644, CI = 0.885, RI = 0.929) which showed the same general topology as that from BI (Fig. 2.1b). All major clades were strongly supported (Wilcox et al. 2002) based on bootstrap values (= 100) and posterior probabilities (= 1.00; Fig. 2.1b). These analyses supported the phylogenetic break between *R. onca* and *R. yavapaiensis* (Jaeger et al. 2001), and further divided *R. yavapaiensis* into two monophyletic clades (with uncorrected *p*-distance = 0.008). One of these clades (herein called the 'main *R. yavapaiensis* clade') represents populations from Arizona and Mexico typically within the uplands of the Sonoran Desert. The other clade represents the single population from Surprise Canyon in the western Grand Canyon (herein called the 'Surprise Canyon population').

Application of substitution rates in BEAST indicate divergence for *R. onca* and *R. yavapaiensis* that most likely occurred around the Early Pleistocene; although the array of molecular rates for the ND2 and Cyt*b* genes results in a broad range for the potential timing of this event (slower rate = 1.95 Mya, 95% CI = 1.42-2.47; faster rate = 0.75 Mya, 95% CI = 0.56-0.96). Divergence of the Surprise Canyon population from the main *R. yavapaiensis* clade appears to have followed around the Middle Pleistocene (slower rate = 0.74 Mya, 95% CI = 0.46-1.05; faster rate = 0.29 Mya 95% CI = 0.18-0.40).

*Population Analyses*

The haplotype network for *R. onca* and *R. yavapaiensis* (Fig. 2.2a) depicted three main groups consistent with the major clades inferred from the MP and BI trees. The two haplotypes of *R. onca* were a minimum of 28 mutational steps within the network from the nearest *R. yavapaiensis* sample from the Surprise Canyon population, and the two haplotypes from the Surprise Canyon population were separated from the main *R. yavapaiensis* group by an additional seven to eight steps. Our ND2 data showed low haplotype and nucleotide diversity within *R. onca* (Table 2.4), consistent with the current population bottleneck.

The main *R. yavapaiensis* clade showed relatively high haplotype diversity (Table 2.4), but the majority of these haplotypes were only a single bp from the common haplotype resulting in a shallow star-shaped pattern (Fig. 2.2a). The most common haplotype (H6) was present at 78% (18/23) of sites (Fig. 2.2b), which affected the assessment of isolation by distance (Mantel test) with only a weak correlation determined between geographic and genetic distances ($r = 0.17$, $P = 0.001$). Many of the *R. yavapaiensis* sites (9/23) were fixed for particular haplotypes, with most of these fixed for the most common haplotype. Visual inspection of haplotype diversities among *R. yavapaiensis* sites showed nearly equal levels across latitudes and elevations indicating no strong correlations with these variables, but this was not surprising given the low genetic diversity within sites (the maximum number of haplotypes at any one site was only three). River basins also appeared to explain only low amounts of genetic variation (Appendix).

The moderately high haplotype diversity coupled with low nucleotide diversity observed within the main *R. yavapaiensis* clade (Table 2.4) indicates the possibility of

rapid population growth (Grant & Bowen 1998; Avise 2000). A signature of growth was also detected from the mismatch distribution assessment which showed a smooth unimodal curve (Fig. 2.3) under the sudden expansion model ($SSD$ = 0.0001, $P$ = 0.949; r = 0.0394, $P$ = 0.828) indicating no significant difference between the observed and simulated pairwise differences. The estimated demographic parameters from the mismatch distribution all indicated sudden expansion (Excoffier & Schneider 1999) since $\tau$ was greater than 0 and $\theta_1 > \theta_0$ ($\tau$ = 1.25, 95% CI = 0.28-2.33; $\theta_1$ = 10.93, 95% CI = 1.45-99,999; $\theta_0$ = 0.035, 95% CI = 0.00-0.55). The time of expansion was indicated to occur around the transition between Middle and Late Pleistocene but with a wide level of uncertainty (slower rate = 0.18 Mya, 95% CI = 0.04-0.33; faster rate = 0.13 Mya, 95% CI = 0.03-0.24). Expansion was also detected in the main *R. yavapaiensis* clade from the significantly negative Fu's $F_S$ (-12.0855; $P$ = 0.001) value and low $R_2$ value (0.0316; $P$ = 0.014) expected from population growth.

*Species Distribution Modeling*

The SDMs for both species produced high training and testing AUC values (Area Under the Curve parameter of the Receiver Operating Characteristic plot; all values ≥ 0.970), indicating that all models performed better than random (Raes & Ter Steege 2007). The SDM for *R. onca* under current climate conditions (Fig. 2.4a) generally represented a reasonable prediction of the known historical distribution as defined by Bradford et al. (2004). The projection of this SDM onto the two Pleistocene climate simulations of the latest glacial maximum produced very different results. The CCSM model (Fig. 2.4b) predicted persistence of potential habitat essentially within the area predicted under current climate along with an unlikely distribution within Death Valley,

California. The MIROC model (Fig. 2.4c), however, predicted an expansion of suitable habitat (along with some overpredictions in areas not likely occupied by these frogs), but importantly this did not extend very far south along the Lower Colorado River or into the Imperial and Mexicali valleys – areas historically occupied by purported *R. yavapaiensis*. Potential habitat was also identified in areas of central Arizona, but this prediction was not always stable under alternative masks used for modeling (data not shown).

For *R. yavapaiensis*, the SDM under current climatic conditions also depicted a reasonable representation of current distribution, but with substantial overprediction of lower probability habitat (Fig. 2.4d). Even with the overprediction, this model did not show substantial overlap with areas occupied by *R. onca*. The projection of the current SDM for *R. yavapaiensis* onto the two Pleistocene climate simulations also produced very different results, although both models predicted a geographic shift towards lower elevation areas of the Sonoran Desert. The model based on CCSM (Fig. 2.4e) predicted a reduction of suitable habitat (particularly higher probability habitat) from that depicted under current conditions, as well as a possible north-south vicariance. The model based on MIROC (Fig. 2.4f) predicted moderate expansion, mostly of lower probability habitat. Importantly, both paleo-SDMs for *R. yavapaiensis* indicated persistence of habitat along the Lower Colorado River extending into the region around the Imperial and Mexicali valleys. Habitat also was predicted in these valleys by SDMs generated for *R. yavapaiensis* that did not include occurrence records from southern California (data not shown).

## Discussion

### *Comparison to Previous Assessments*

Our assessment corroborates the previously determined phylogenetic break between *R. onca* and *R. yavapaiensis* (Jaeger et al. 2001), as we found no admixing of *R. onca* and *R. yavapaiensis* haplotypes within sites after extensive sampling. However, our analyses indicate a more complex history for these frogs than previously supposed (Jaeger et al. 2001), and our phylogeographic assessment further divided *R. yavapaiensis* into two distinct mtDNA lineages – one representing populations across the main range in Arizona and northern Mexico, and the other representing the disjunct population in the western Grand Canyon.

Jaeger et al. (2001) suggested that the level of mtDNA divergence between *R. onca* and *R. yavapaiensis* represented Late Pleistocene-Holocene isolation, but our divergence estimates indicate the possibility of an older timing for this separation, possibly dating to around the Early Pleistocene. Further, under the assumption that our molecular clocks are moderately accurate, the shallow divergence of the Surprise Canyon population from the main clade of *R. yavapaiensis* appears to date to the Middle Pleistocene. These molecular clock interpretations, however, must be viewed speculatively, as demographic and selective processes can greatly influence the coalescence of mtDNA, resulting in deeper phylogenetic separation than warranted by actual divergence time (Avise 2000). One possibility is that the observed patterns could have been caused by an overall decline in a highly diverse ancestral (*R. onca-yavapaiensis*) species that left behind small regional populations that retained, and then fixed divergent ancestral polymorphisms. This may be more common in organisms, such as these frogs, in which regional dispersal is perhaps limited, population size fluctuates greatly (lowering $N_e$), and selective sweeps may be an important evolutionary factor; for example in anurans (and other ectotherms)

temperature directly impacts the mitochondria and changes in this climatic feature may lead to selection favoring particular genotypes (Ballard & Whitlock 2004).

Demographic patterns that could have affected interpretations of divergence timing are clearly evident in these species. The Surprise Canyon population of *R. yavapaiensis* currently appears to be isolated in one drainage within the western Grand Canyon (CAD, JRJ, and DFB unpublished data), and *R. onca* has suffered a dramatic, recent decline (Bradford et al. 2004). The low genetic diversity observed in *R. onca* was expected given its overall decline, and was consistent with a previous assessment of nuclear genetic diversity based on randomly amplified polymorphic DNA (RAPD) data (Jaeger et al. 2001). It is also possible that *R. onca* may have always been geographically limited (as depicted in one paleo-SDM; Fig. 2.4b), and even if it was more broadly distributed our genetic sampling represents only the few remaining, closely situated populations.

For *R. yavapaiensis,* the genetic data indicate that the main clade has historically undergone population expansion. Moderately high haplotype diversity coupled with low nucleotide diversity within the *R. yavapaiensis* clade indicates the possibility of a population bottleneck followed by rapid growth (Grant & Bowen 1998; Avise 2000). Support for an interpretation of population expansion comes from the mismatch distribution assessment and from the neutrality test results. This signal of expansion in *R. yavapaiensis* might be attributable to population or range expansion following the latest glacial period, as depicted by the difference between the current SDM (Fig. 2.4d) and one of the paleo-SDMs (Fig. 2.4e). However, a rough estimate of the time of this expansion, derived from the assessment of mismatch distribution, suggests a time frame that likely predates the recent glacial maximum. Importantly, genetic diversity across the core *R.*

*yavapaiensis* distribution shows no strong correlation with latitude, thus providing no evidence for the commonly envisioned pattern of northward expansions of warm-adapted species from glacial refugia in more southern areas of the Sonoran Desert. Instead, the genetic pattern is consistent with an interpretation that *R. yavapaiensis* responded with only moderate shifts in distributions during the last glacial period mostly to adjacent areas of lower elevation (Fig. 2.4e, 2.4f).

*Biogeographic Patterns*

A likely scenario for the phylogeographic patterns observed for *R. onca* and *R. yavapaiensis*, particularly along the Colorado River, is that the ancestral lineage to these frogs expanded and contracted multiple times (at least twice) during the Quaternary, probably from the core areas identified for *R. yavapaiensis* within the northern Sonoran Desert, essentially allowing connections to the Colorado River. This was followed by contractions of the main population and subsequent isolation and divergence of remnant populations within northern, or possibly western, refugia. *Rana onca* may have subsequently evolved as a local endemic, restricted to a narrow area along the Colorado River and its tributaries within the eastern Mojave Desert (Fig. 2.4a). *Rana yavapaiensis*, on the other hand, is associated with areas identified as Sonoran Desert, including areas along the Lower Colorado River and the Imperial and Mexicali valleys (Fig. 2.4d). Assuming local adaptation, differences in the climates between these desert regions may have contributed to limiting long term contact between these taxa.

The disjunct location of the Surprise Canyon population of *R. yavapaiensis* may seem hard to explain, given that *R. onca* populations occupy the Colorado River corridor between Surprise Canyon and populations of *R. yavapaiensis* along the Lower Colorado

River. However, the nearest population of *R. yavapaiensis* to Surprise Canyon is in Willow Creek, about 85 km due south (site WC in Fig. 2.1a), and there is a relatively low divide between the headwaters of this drainage and the north-flowing tributaries that feed into the Colorado River in the vicinity of Surprise Canyon. Much of the upper parts of these drainages are dry under current climatic conditions, but we suspect that this was a likely pathway that once connected the main distribution of *R. yavapaiensis* with Surprise Canyon under a cooler or wetter climate. What is striking is that the Surprise Canyon population shows a level of divergence that indicates longevity to its isolation. There is, however, evidence from paleo-reconstructions that lower elevations of the western Grand Canyon retained warmer conditions through the last glacial maximum (e.g. Phillips 1977). This could have allowed persistence of these frogs through time within an isolated northern refugium in the canyon region (one not depicted by our coarse-scale paleo-SDMs).

## Conclusions

The main phylogeographic patterns observed for *R. onca* and *R. yavapaiensis* are likely robust at the organismal level and expand our understanding of the evolutionary history of this group. Given the observed levels of mtDNA divergence and previous research that included nuclear (RAPD) and morphological assessments which supported the main divergence (Jaeger et al. 2001), the further application of nuclear genes are not likely to change the interpretation of these patterns, as many of these genes would not be expected to track this more recent evolutionary history (e.g. Zink & Barrowclough 2008). Of more importance to interpretations of the phylogeography of *R. onca* and *R.*

*yavapaiensis* would be a genetic assessment of historical (museum) specimens from extirpated populations in southern California.

Our data point to the uniqueness of the northernmost population of *R. yavapaiensis* within Surprise Canyon. While the level of difference from other *R. yavapaiensis* populations based on mtDNA may not warrant taxonomic recognition at this time, this disjunct population merits conservation consideration and further study. Finally, the tentative identification of *R. magnaocularis* haplotypes at sites in Sonora thought to contain *R. yavapaiensis* indicates a need to refine our understanding of the distributions and genetic structure (including the possibility of hybridization) of these species in Mexico.

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**Table 2.1.** Exemplar samples of ND2 haplotypes for *Rana onca* (H1-2), *R. yavapaiensis* (H3-23), and tentatively identified *R. magnaocularis* (M1-6). For phylogeographic analysis, each sample was also sequenced for Cyt*b*. Exemplar samples are listed by sample number, site, county, state, and country. Further information on locations is available in Table 2.2. Outgroup samples of *R. forreri* and *R.* 'species 8' are identified by sample number and type locality. Sequences are available from GenBank under accession numbers GU184190-GU184251.

| Haplotype Number | Sample Number | Type Locality |
|---|---|---|
| H1 | LVT3541 | Bighorn Sheep Spring, Clark Co., NV, USA |
| H2 | LVT3440 | Blue Point Spring, Clark Co., NV, USA |
| H3 | LVT7091 | Surprise Canyon, Mohave, Co., AZ, USA |
| H4 | LVT7095 | Surprise Canyon, Mohave, Co., AZ, USA |
| H5 | LVT4560 | Trout Creek, Mohave, Co., AZ, USA |
| H6 | LVT9531 | Río Cocospera, Rancho el Aribabi, SO, MX |
| H7 | LVT4562 | Trout Creek, Mohave, Co., AZ, USA |
| H8 | LVT4579 | Trout Creek, Mohave, Co., AZ, USA |
| H9 | LVT8814 | Santa Maria River, Yavapai Co., AZ, USA |
| H10 | LVT4567 | Cottonwood Creek, Yavapai Co., AZ, USA |
| H11 | LVT8092 | Coon Creek, Gila Co., AZ, USA |
| H12 | LVT8037 | Pinto Creek, Gila Co., AZ, USA |
| H13 | LVT7395 | Aravaipa Creek, Graham Co., AZ, USA |
| H14 | LVT8181 | Markham Creek, Graham Co., AZ, USA |
| H15 | LVT7190 | Muleshoe Hotsprings, Cochise Co., AZ, USA |
| H16 | LVT7983 | Cienega Creek, Santa Cruz Co., AZ, USA |
| H17 | LVT9548 | Alamo Canyon, Santa Cruz Co., AZ, USA |
| H18 | LVT9534 | Río Cocospera, Rancho el Aribabi, SO, MX |
| H19 | LVT9532 | Río Cocospera, Rancho el Aribabi, SO, MX |
| H20 | NK3927 | Canon Bonito, Rancho Nuevo, SO, MX |
| H21 | NK3929 | Canon Bonito, Rancho Nuevo, SO, MX |
| H22 | LVT9990 | Canon el Pulpito, SO, MX |
| H23 | LVT9015 | Río Tutuaca, Rancho el Nogal, CH, MX |
| M1 | LVT9501 | Río Yaqui, SO, MX |
| M2 | LVT9970 | Río Sahuaripa, SO, MX |
| M3 | LVT9521 | Río Sonora, SO, MX |
| M4 | LVT10354 | Arroyo San Ignacio, SO, MX |
| M5 | LVT9503 | Río Yaqui, SO, MX |
| M6 | LVT10353 | Arroyo San Ignacio, SO, MX |
| *R. forreri* | KU194581 | 37.9 km S. of Escuinapa, SI, MX |
| *R.*'species 8' | KU195346 | Río Atoyac at Mexico Hwy. 190, PU, MX |

**Table 2.2.** Sample sites for *Rana onca* and *R. yavapaiensis* by county, state, country, site labels (referenced in figures), geographic coordinates (datum NAD27), and haplotypes observed. Also shown are sites in Sonora where samples have been tentatively identified as *R. magnaocularis*.

| Species, Site, County, State, Country | Label | Lat. | Long. | Haplotype (*n*) |
|---|---|---|---|---|
| *Rana onca* | | | | |
| Bighorn Sheep Spring, Clark Co., NV, USA | BH | 35.939 | -114.733 | H1(10) |
| Blue Point Spring, Clark Co., NV, USA | BP | 36.389 | -114.432 | H2(10) |
| Boy Scout Canyon, Clark Co., NV, USA | BS | 35.984 | -114.745 | H1(10) |
| Littlefield, Mohave Co., AZ, USA | LF | 36.908 | -113.896 | H1(10) |
| Rogers Spring, Clark Co, NV, USA | RS | 36.378 | -114.443 | H2(4) |
| Salt Cedar Canyon, Clark Co., NV, USA | SC | 35.965 | -114.743 | H1(7) |
| *Rana yavapaiensis* | | | | |
| Alamo Canyon, Santa Cruz Co., AZ, USA | AC | 31.365 | -111.135 | H17(8) |
| Aliso Spring, Santa Cruz Co., AZ, USA | AS | 31.581 | -111.099 | H6(10) |
| Aravaipa Creek, Graham Co., AZ, USA | AR | 32.878 | -110.392 | H6(1), H13(9) |
| Canon Bonito, Rancho Nuevo, SO, MX | RN | 31.232 | -108.920 | H6(1), H20(3), H21(1) |
| Canon el Pulpito, SO, MX | CP | 30.777 | -109.005 | H20(4), H22(6) |
| Cienega Creek, Santa Cruz Co., AZ, USA | CN | 32.011 | -110.623 | H16(3), H17(4) |
| Coon Creek, Gila Co., AZ, USA | CR | 33.686 | -110.843 | H6(9), H11(1) |
| Cottonwood Creek, Yavapai Co., AZ, USA | CC | 33.903 | -112.324 | H6(8), H10(2) |
| Hassayampa R., Maricopa Co., AZ, USA | HA | 33.931 | -112.692 | H6(10) |
| Kayler Spring, Gila Co., AZ, USA | KS | 33.945 | -111.302 | H6(8) |
| Markham Creek, Graham Co., AZ, USA | MC | 33.091 | -109.823 | H14(10) |
| Mineral Creek, Pinal Co., AZ, USA | MN | 33.251 | -110.983 | H6(1), H11(5) |
| Muleshoe Hotspr., Cochise Co., AZ, USA | MH | 32.338 | -110.250 | H6(10), H15(2) |
| Pinto Creek, Gila Co., AZ, USA | PC | 33.457 | -111.005 | H6(1), H12(9) |
| Río Bavispe, near Huachinera, SO, Mexico | RB | 30.205 | -108.957 | H6(10) |
| Río Cocospera, Rancho el Aribabi, SO, MX | RC | 30.858 | -110.663 | H6(2), H18(6), H19(2) |
| Río Tutuaca, Rancho el Nogal, CH, MX | RE | 28.560 | -108.356 | H6(1), H23(5) |

| | | | | |
|---|---|---|---|---|
| Santa Maria River, Yavapai Co., AZ, USA | SM | 34.368 | -113.184 | H6(10), H9(1) |
| Sheep Wash, Greenlee Co., AZ, USA | SW | 33.303 | -109.404 | H6(9) |
| Surprise Canyon, Mohave Co., AZ, USA | SU | 35.908 | -113.620 | H3(15), H4(8) |
| Tonibabi, SO, MX | TB | 29.833 | -109.562 | H6(10) |
| Trout Creek, Mohave Co., AZ, USA | TC | 35.000 | -113.447 | H5(4), H7(2), H8(2) |
| Turkey Creek, Greenlee Co., AZ, USA | TU | 33.288 | -109.261 | H6(7) |
| Willow Creek, Mohave Co., AZ, USA | WC | 35.144 | -113.530 | H5(3), H6(2) |
| *Rana magnaocularis* | | | | |
| Arroyo San Ignacio, SO, MX | SI | 28.699 | -109.085 | M1(2), M4(2), M5(1), M6(1) |
| Río Sahuaripa, SO, MX | SR | 29.186 | -109.277 | M1(5), M2(3), M4(1), M5(1) |
| Río Sonora, SO, MX | SN | 29.331 | -110.537 | M3(10) |
| Río Yaqui, SO, MX | RY | 28.591 | -109.560 | M1(7), M5(3) |

**Table 2.3.** Sources for observation records of *Rana onca* and *R. yavapaiensis* used in species distribution modeling.

| Species | Data Type | Source |
|---|---|---|
| *R. onca* | Literature | Bradford *et al*., 2004 |
| | Museum Records | California Academy of Sciences, San Francisco |
| | | Carnegie Museum of Natural History, Pittsburgh |
| | | Los Angeles County Museum of Natural History, Los Angeles |
| | | Marjorie Barrick Museum of Natural History, University of Nevada, Las Vegas |
| | | Monte L. Bean Life Science Museum, Brigham Young University, Provo |
| | | Museum of Vertebrate Zoology, University of California, Berkeley |
| *R. yavapaiensis* | Literature | Jennings, 1995 |
| | Museum Records | Museum of Vertebrate Zoology, University of California, Berkeley |
| | | Museum of Natural History, University of Arizona, Tucson |
| | Database | Ranid Frog Database – Arizona Game and Fish Department, Phoenix |
| California records | Literature | Jennings & Hayes, 1994 |
| | Museum Records | Louisiana Museum of Natural History, Baton Rouge |
| | | Smithsonian National Museum of Natural History, Washington, D.C. |

**Table 2.4.** Molecular diversity indices for ND2 sequences of *Rana onca*, the main clade of *R. yavapaiensis*, the Surprise Canyon population of *R. yavapaiensis*, and all *R. yavapaiensis* samples combined. Shown are sample sizes (*n*), numbers of haplotypes (*nh*), haplotype diversity with standard error (*h* ± SE), and nucleotide diversity with standard error ($\pi$ ± SE).

| **Taxon** | ***n*** | ***nh*** | ***h* ± SE** | **$\pi$ ± SE (x100)** |
|---|---|---|---|---|
| *R. onca* | 51 | 2 | 0.4063±0.0575 | 0.0393±0.0409 |
| Main *R. yavapaiensis* | 202 | 19 | 0.6905±0.0357 | 0. 1164±0.0826 |
| Surprise Canyon | 23 | 2 | 0.4743±0.0668 | 0.0458±0.0461 |
| All *R. yavapaiensis* | 225 | 21 | 0.7454±0.0302 | 0.2418±0.1448 |

**Figure 2.1. (a)** Sampled sites for genetic analysis with location abbreviations from Table 2.2. Circle shading reference taxa as follows: *Rana onca* (black); Surprise Canyon population of *R. yavapaiensis* (dark gray); *R. yavapaiensis* (light gray); and tentatively identified *R. magnaocularis* from locations originally sampled for *R. yavapaiensis* (white). Circle size is proportional to sample size (largest = 23, smallest = 4). **(b)** Depiction of the phylogenetic relationship of *R. onca* and *R. yavapaiensis* haplotypes based on 50% majority-rule consensus tree (ln L = -5283.75) from Bayesian inference runs. All major nodes are supported by 100% Bayesian inference posterior probabilities and Maximum parsimony bootstrap values (shown).

**Figure 2.2. (a)** Median-joining haplotype network of *Rana onca* and *R. yavapaiensis* with haplotypes coded by number. Crossbars along connection lines indicate a mutational change; the white square represents either an unsampled or an extinct common ancestor haplotype. Haplotypes are identified by shading according to the three major clades depicted in Fig. 2.1b. Circle size reflects the number of sampled individuals sharing a haplotype (largest = 110, smallest = 1). **(b)** The geographic distribution of ND2 haplotypes of *R. onca* and *R. yavapaiensis*. Haplotypes are referenced by code as depicted in the network, and pie size reflects the number of individuals per haplotype at each site.

**Figure 2.3.** Mismatch distribution analysis of ND2 sequence data from the main *Rana yavapaiensis* clade (excluding the Surprise Canyon samples) under the sudden expansion model.

**Figure 2.4.** Species distribution models for *Rana onca* under current climate conditions (a) and two glacial models, CCSM (b) and MIROC (c), and *R. yavapaiensis* under current climate (d), CCSM (e) and MIROC (f). White dots indicate sample locations. Higher (dark gray) and lower (lighter gray) logistic probability values for predicted suitable habitats are depicted.

**Figure2.1.**

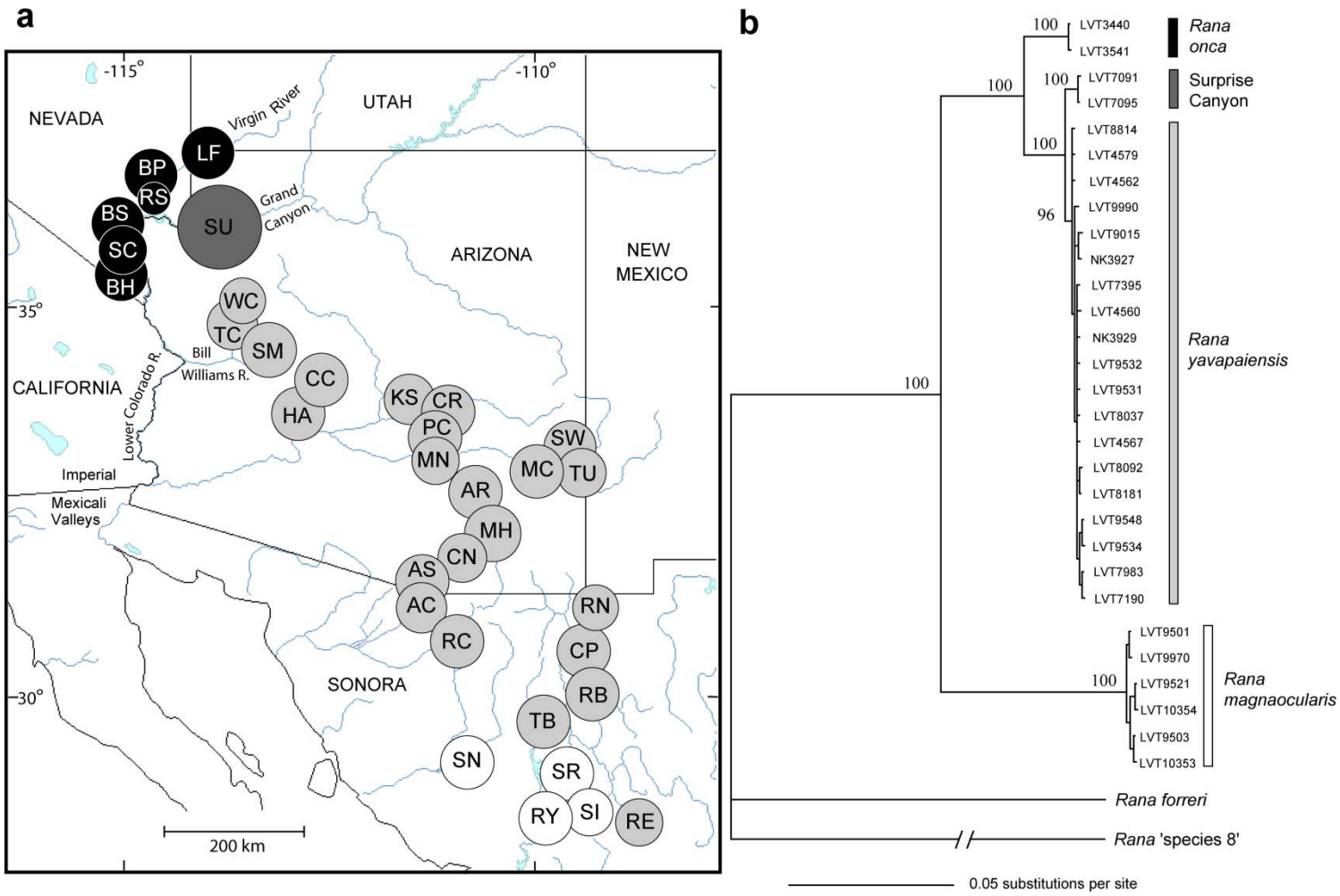

a
-115°
-110°
NEVADA
UTAH
ARIZONA
NEW MEXICO
CALIFORNIA
SONORA
Virgin River
Grand Canyon
Bill Williams R.
Lower Colorado R.
Imperial
Mexicali Valleys
35°
30°
200 km
LF
BP
RS
BS
SC
BH
SU
WC
TC
SM
CC
HA
KS
CR
PC
MN
AR
MH
CN
AS
AC
RC
SW
MC
TU
RN
CP
RB
TB
SN
SR
RY
SI
RE
b
LVT3440
LVT3541
LVT7091
LVT7095
LVT8814
LVT4579
LVT4562
LVT9990
LVT9015
NK3927
LVT7395
LVT4560
NK3929
LVT9532
LVT9531
LVT8037
LVT4567
LVT8092
LVT8181
LVT9548
LVT9534
LVT7983
LVT7190
LVT9501
LVT9970
LVT9521
LVT10354
LVT9503
LVT10353
100
96
Rana onca
Surprise Canyon
Rana yavapaiensis
Rana magnaocularis
Rana forreri
Rana 'species 8'
0.05 substitutions per site

**Figure 2.2.**

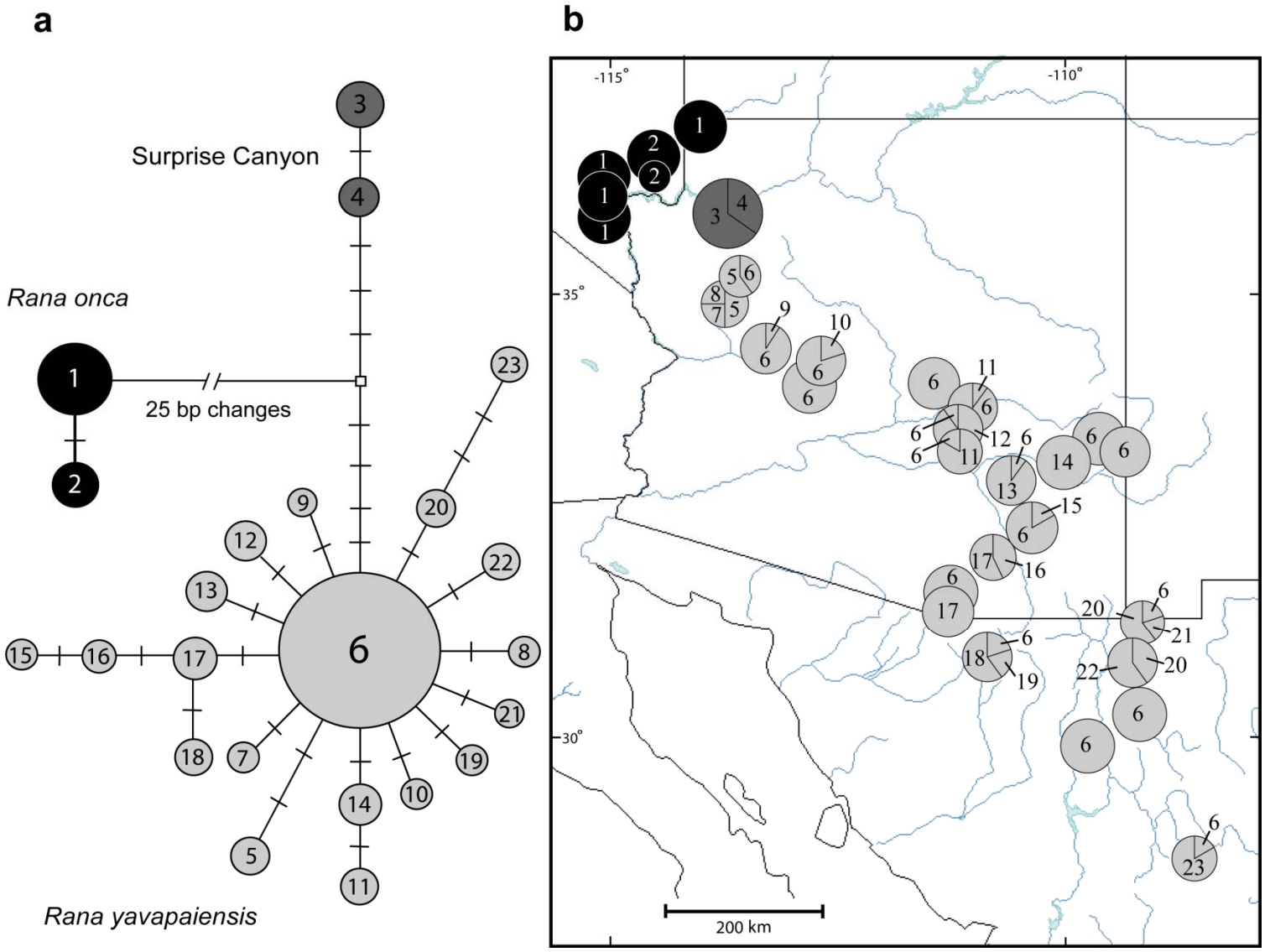

a
b
Surprise Canyon
Rana onca
25 bp changes
Rana yavapaiensis
-115°
-110°
35°
30°
200 km

**Figure 2.3.**

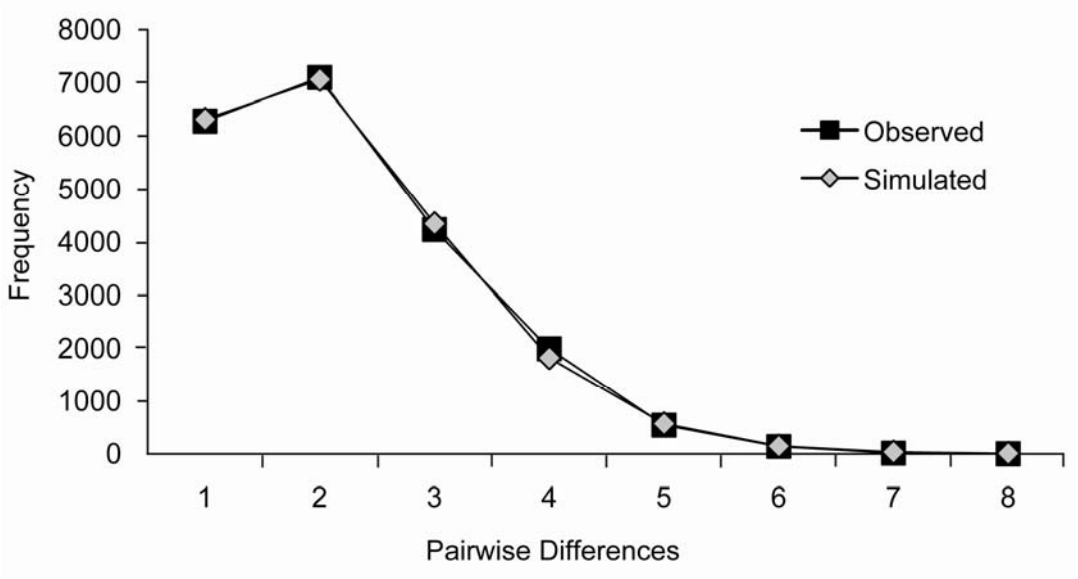
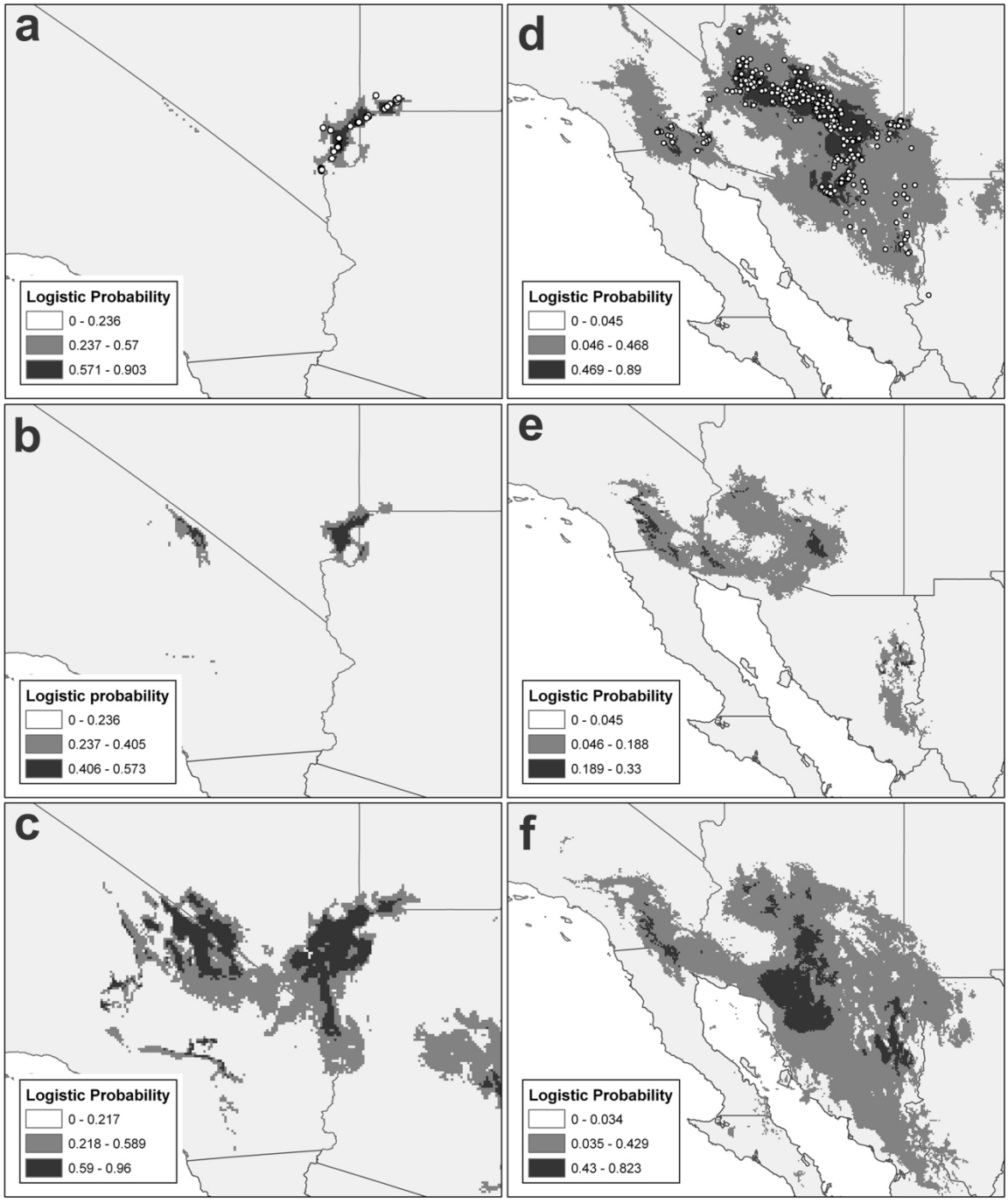

8000
7000
6000
5000
4000
3000
2000
1000
0
Frequency
1
2
3
4
5
6
7
8
Pairwise Differences
Observed
Simulated

**Figure 2.4.**


a
Logistic Probability
0 - 0.236
0.237 - 0.57
0.571 - 0.903
d
Logistic Probability
0 - 0.045
0.046 - 0.468
0.469 - 0.89
b
Logistic probability
0 - 0.236
0.237 - 0.405
0.406 - 0.573
e
Logistic Probability
0 - 0.045
0.046 - 0.188
0.189 - 0.33
c
Logistic Probability
0 - 0.217
0.218 - 0.589
0.59 - 0.96
f
Logistic Probability
0 - 0.034
0.035 - 0.429
0.43 - 0.823

## Appendix

### Assessment of genetic variation in *Rana yavapaiensis* among river basins

#### Materials and Methods

We assessed genetic variation of ND2 among river basins by conducting an analysis of molecular variance (AMOVA) in ARLEQUIN (10,000 permutations; pairwise difference distances). Within the USA, we grouped sample sites along the Bill Williams, Gila, Upper Gila, Salt, and Santa Cruz rivers by basins using 8 digit Hydrologic Unit Codes (HUCs; U.S. Geological Survey). We grouped sites across HUCs along the Middle Gila and San Pedro rivers that shared contiguous stretches of perennial water. Because no system comparable to 8 digit HUCs exists for Mexico, we grouped sites by major river basins and proximity based on 1:200,000 maps (Table A1.).

#### Results

River basins explained a significant, although low amount of the total genetic variation (12.8%). Most genetic variation (51.8%) occurred among sites within river basins, likely because of the relatively high levels of fixation within these sites (fixation indices $\Phi_{SC} = 0.594$, $\Phi_{ST} = 0.646$, and $\Phi_{CT} = 0.128$, all $P \leq 0.03$).

#### Discussion

Our assessments of haplotype distribution and diversity suggest that current environmental conditions may limit regional dispersal of *R. yavapaiensis* among river basins despite a signal of older expansion. While little genetic structure was attributable

to river basins (consistent with and interpretation of high gene flow), this pattern was influenced by the persistence of the most common haplotype in high frequencies across the entire range. Most unique haplotypes are restricted to single or nearby sites and not shared among river basins (Fig. 2.2b) suggesting the possibility that the period of expansion was followed by more recent restricted levels of migration and gene flow among regional populations.

**Table A1.** Number of *Rana yavapaiensis* samples (*n*) grouped by river basins for AMOVA. Site labels reference Fig. 2.1a and Table 2.2.

| Group | Basin | Sites by Label | *n* |
|---|---|---|---|
| 1 | Bill Williams River | WC, TC, SM | 24 |
| 2 | Lower Gila River | HA, CC | 20 |
| 3 | Middle Gila & San Pedro rivers | MN, AR, MH | 28 |
| 4 | Upper Gila River | MC, SW, TU | 26 |
| 5 | Salt River | KS, CR, PC | 28 |
| 6 | Santa Cruz River | CN, AS, AC | 25 |
| 7 | Río Concepcion | RC | 10 |
| 8 | Río Bavispe | CP, RN, RB | 25 |
| 9 | Río Moctezuma | TB | 10 |
| 10 | Río Tutuaca | RE | 6 |