

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* (= *Lithobates onca*) and lowland leopard frog *Rana yavapaiensis* (= *Lithobates yavapaiensis*) – two declining anurans from the warm-desert regions of south-western North America. We used sequence data from mitochondrial DNA (mtDNA) to assess 276 individuals representing 30 sites from across current distributions. Our analysis supports a 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 phylogeographic 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 core 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.

Introduction

The relict leopard frog, *Rana onca* (= *Lithobates onca*) and the lowland leopard frog, *Rana yavapaiensis* (= *Lithobates yavapaiensis*), occupy springs, streams and wetlands within warm-desert regions of south-western 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 south-western Utah, north-western 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. 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

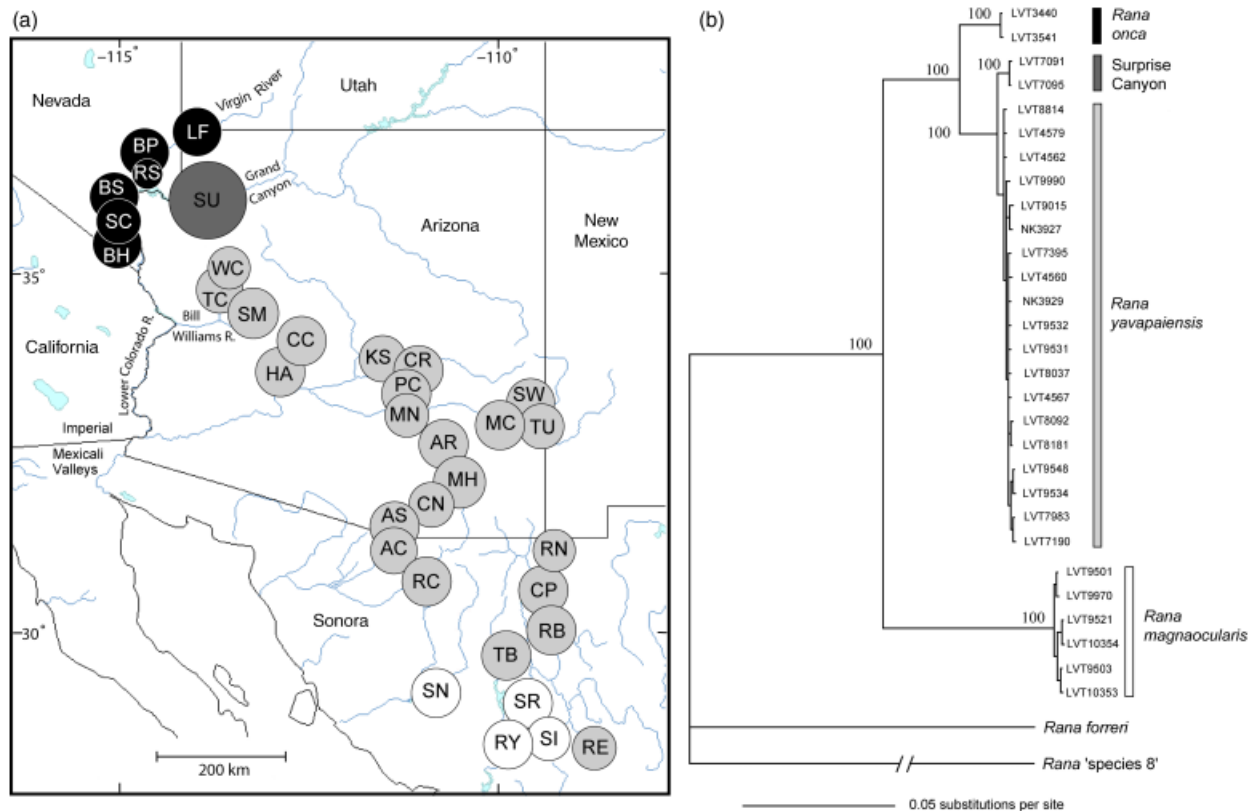


Figure 1 (a) Sampled sites for genetic analysis with location abbreviations from supporting information Table S1. Circle shading reference taxa as follows: *Rana onca* (black); Surprise Canyon population of *Rana yavapaiensis* (dark gray); *R. yavapaiensis* (light gray); and tentatively identified *Rana 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).

(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. 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. 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 considerably affected environmental conditions in these deserts (e.g. Betancourt, Van Devender & Martin, 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, Hafner & Alexander, 2000; Douglas *et al.*, 2006; Fehlberg & Ranker, 2009). For example, low mtDNA diversity in populations of the red-spotted toad *Bufo punctatus* within the north-eastern 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, Riddle & Bradford, 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. 1a; Table 1, see supporting information Table S1). These samples included: 51 *R. onca* from five sites in southern Nevada and one site in north-western Arizona (the LF site in Fig. 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 *Rana magnaocularis* (Frost & Bagnara, 1976). We used samples of *Rana 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

Table 1 Exemplar samples of ND2 haplotypes for *Rana onca* (H1–2), *Rana yavapaiensis* (H3–23), and tentatively identified *Rana magnaocularis* (M1–6)

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	Rio Cocospera, Rancho el Aribabi, Sonora, Mexico
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	Rio Cocospera, Rancho el Aribabi, Sonora, Mexico
H19	LVT9532	Rio Cocospera, Rancho el Aribabi, Sonora, Mexico
H20	NK3927	Canon Bonito, Rancho Nuevo, Sonora, Mexico
H21	NK3929	Canon Bonito, Rancho Nuevo, Sonora, Mexico
H22	LVT9990	Canon el Pulpito, Sonora, Mexico
H23	LVT9015	Rio Tutuaca, Rancho el Nogal, Chihuahua, Mexico
M1	LVT9501	Rio Yaqui, Sonora, Mexico
M2	LVT9970	Rio Sahuaripa, Sonora, Mexico
M3	LVT9521	Rio Sonora, Sonora, Mexico
M4	LVT10354	Arroyo San Ignacio, Sonora, Mexico
M5	LVT9503	Rio Yaqui, Sonora, Mexico
M6	LVT10353	Arroyo San Ignacio, Sonora, Mexico
<i>Rana forreri</i>	KU194581	37.9 km S. of Escuinapa, Sinaloa, Mexico
<i>R. 'species 8'</i>	KU195346	Rio Atoyac at Mexico highway. 190, Puebla, Mexico

For phylogeographic analysis, each sample was also sequenced for Cytb. Exemplar samples are listed by sample number, site, county, state and country (USA or Mexico). Further information on locations is available in supporting information Table S1. Outgroup samples of *R. forreri* and *R. 'species 8'* are identified by sample number and location. Sequences are available from GenBank under accession numbers GU184190–GU184251.

cytochrome *b* (Cyt *b*). We used primers L3880 and H6033 (Riddle, Honeycut & Lee, 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'-GAAATTCCTTGAAG GACCTCAGG-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 and 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, Laval & Schneider, 2005) and mean pairwise sequence divergences (uncorrected *p*-distances) using MEGA v. 4 (Tamura *et al.*, 2007). Before 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 using 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 first + second codon positions and the General Time Reversible (GTR) model for the third 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 (MCMC) 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 three 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 used 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⁻¹ year⁻¹ (s s⁻¹ y⁻¹), 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⁻¹ y⁻¹). A much faster rate of 3.6% ($\mu = 1.8 \times 10^{-8}$ s s⁻¹ y⁻¹) 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). Before estimation, we tested the concatenated (haplotype) dataset 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 1st + 2nd codon positions and GTR for the 3rd 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 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 dataset of our taxa using a median-joining network (Bandelt, Forster & Röhl, 1999) constructed in NETWORK v. 4.2.0.1 (<http://www.fluxus-engineering.com>). We evaluated isolation by distance among sites (pairwise *F*_{st} values vs. 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 τ (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 μ is the per-sequence mutation rate per generation (Rogers & Harpending, 1992). We assumed ND2 rates of both 7.1×10^{-6} and 9.9×10^{-6} substitutions locus⁻¹ year⁻¹ (from above) and a 2-year generation time for female *R. yavapaiensis* (Sredl, Collins & Howland, 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).

SDM

We used the program MAXENT v. 3.3.1 (Phillips, Anderson & Schapire, 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 (<http://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 (see supporting information Table S2). Our genetic sampling, however, revealed frogs with divergent mtDNA at four locations purported to be *R. yavapaiensis* sites in southern Sonora (Fig. 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 > 5 km. The final dataset 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 (c. 21 000 BP) 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* (north-west corner 38.25°, –118.67°; south-east corner 31.46°, –111.50°) and *R. yavapaiensis* (north-west corner 38.04°, –118.63°; south-east corner 25.50°, –105.63°). Habitat suitability was displayed as two categories in ArcGIS v.9.2. (ESRI Inc., Redlands, CA, USA, 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 two *R. onca* and 21 *R. yavapaiensis* haplotypes for which we generated additional Cyt *b* data on exemplars (Table 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. 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 *Rana 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 & 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 & Markow (2008), further assessments are necessary clarifying the phylogenetic and taxonomic relationships among leopard frogs in the region.

MP analysis of the concatenated dataset resulted a single tree (length = 644, CI = 0.885, RI = 0.929), which showed the same general topology as that from BI (Fig. 1b). All major clades were strongly supported (Wilcox *et al.*, 2002) based on bootstrap values (= 100) and posterior probabilities (= 1.00; Fig. 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. 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

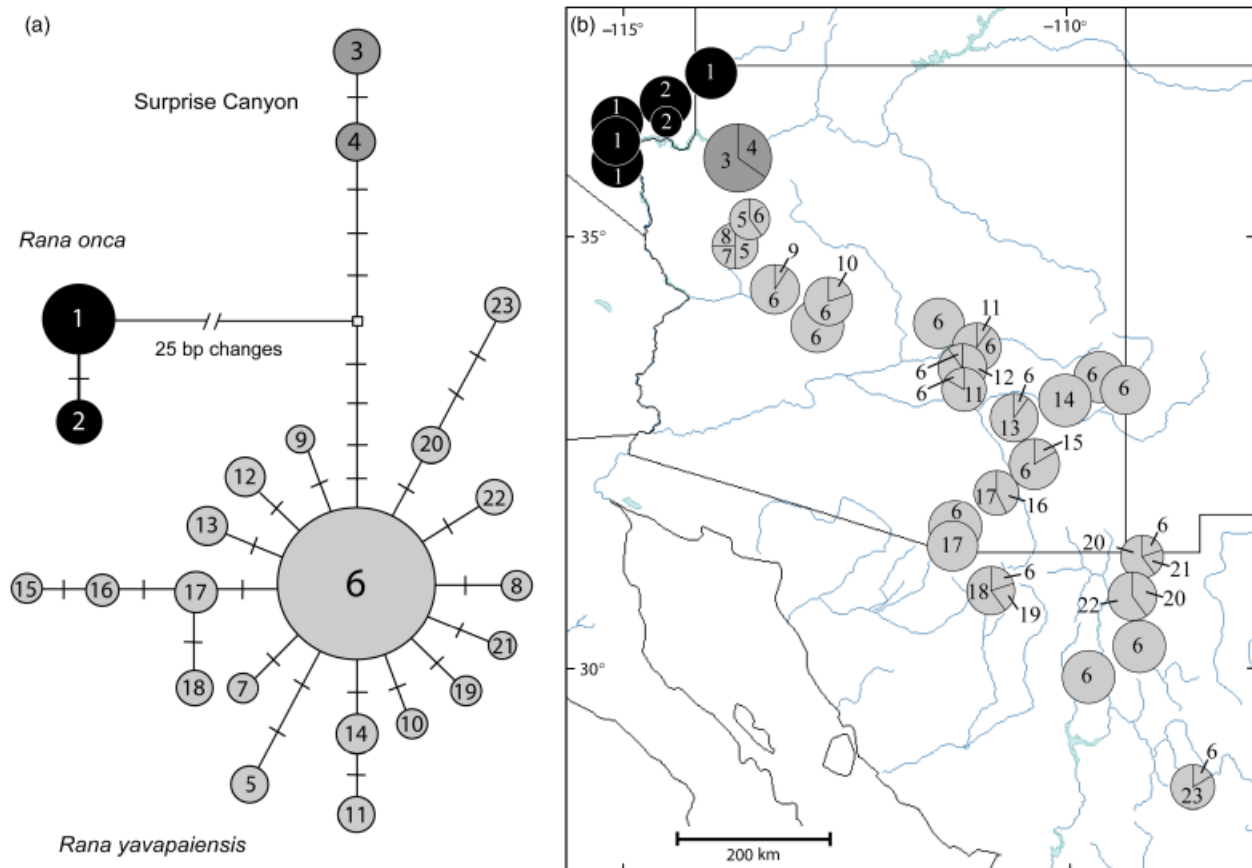


Figure 2 (a) Median-joining haplotype network of *Rana onca* and *Rana yavapaiensis* with haplotypes coded by number. Crossbars along connection lines indicate a mutational change; the white square represents either an unsampled or an extinct common ancestor haplotype. Haplotypes are identified by shading according to the three major clades depicted in Fig. 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.

haplotype and nucleotide diversity within *R. onca* (Table 2), consistent with the current population bottleneck.

The main *R. yavapaiensis* clade showed relatively high haplotype diversity (Table 2), but the majority of these haplotypes were only a single bp from the common haplotype resulting in a shallow star-shaped pattern (Fig. 2a). The most common haplotype (H6) was present at 78% (18/23) of sites (Fig. 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 (see supporting information Appendix S1).

The moderately high haplotype diversity coupled with low nucleotide diversity observed within the main *R. yavapaiensis* clade (Table 2) 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 (see supporting information Fig. S1) 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 τ was > 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.

Table 2 Molecular diversity indices for ND2 sequences of *Rana onca*, the main clade of *Rana yavapaiensis*, the Surprise Canyon population of *R. yavapaiensis* and all *R. yavapaiensis* samples combined

Taxon	<i>n</i>	<i>n_h</i>	<i>h</i> ± SE	π ± SE (× 100)
<i>Rana onca</i>	51	2	0.4063 ± 0.0575	0.0393 ± 0.0409
Main <i>Rana yavapaiensis</i>	202	19	0.6905 ± 0.0357	0.1164 ± 0.0826
Surprise Canyon	23	2	0.4743 ± 0.0668	0.0458 ± 0.0461
All <i>Rana yavapaiensis</i>	225	21	0.7454 ± 0.0302	0.2418 ± 0.1448

Shown are sample sizes (*n*), numbers of haplotypes (*n_h*), haplotype diversity with standard error (*h* ± SE), and nucleotide diversity with standard error (π ± SE).

SDM

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. 3a) 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. 3b) 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. 3c), 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. 3d). 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. 3e) 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. 3f) 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

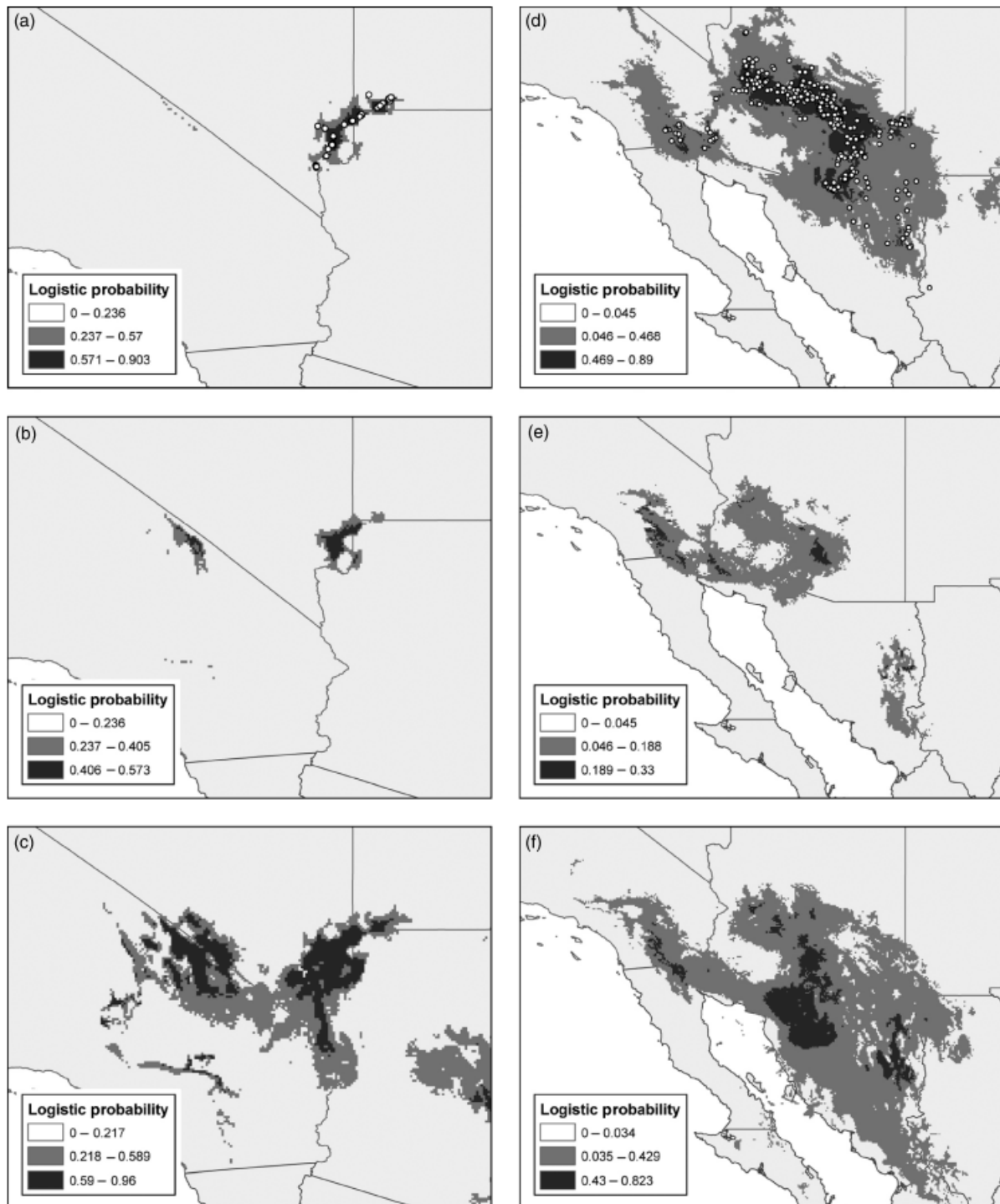


Figure 3 Species distribution models for *Rana onca* under current climate conditions (a) and two glacial models, CCSM (b) and MIROC (c) and *R. yavapaiensis* under current climate (d) CCSM (e) and MIROC (f). White dots indicate sample locations. Higher (dark grey) and lower (lighter grey) logistic probability values for predicted suitable habitats are depicted.

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 (Avice, 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 considerably (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 (C. A. Drost, J. R. Jaeger, and D. F. Bradford, unpubl. 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. 3b), 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 indicate the possibility of a population bottleneck followed by rapid growth (Grant & Bowen, 1998; Avice, 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. 3d) and one of the paleo-SDMs (Fig. 3e). 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. 3e and f).

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. 3a). *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. 3d). 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. 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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Supporting Information

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

Table S1. Sample sites for *Rana onca* and *Rana yavapaiensis* by state (Mexico or USA), site labels (referenced in figures), geographic coordinates (datum NAD27), and haplotypes observed.

Table S2. Sources for observation records used in species distribution modeling of *Rana onca* and *Rana yavapaiensis*.

Appendix S1. Assessment of genetic variation in *Rana yavapaiensis* among river basins.

Figure S1. Mismatch distribution analysis of ND2 sequence data from the main *Rana yavapaiensis* clade (excluding the Surprise Canyon samples) under the sudden expansion model.

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