Rediscovering *Rana onca*: Evidence for Phylogenetically Distinct Leopard Frogs from the Border Region of Nevada, Utah, and Arizona

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Remnant populations of leopard frogs within the Virgin River drainage and adjacent portions of the Colorado River (Black Canyon) in northwestern Arizona and southern Nevada either represent the reportedly extinct taxon *Rana onca* or northern, disjunct *Rana yavapaiensis*. To determine the evolutionary distinctiveness of these leopard frogs, we evaluated mitochondrial DNA (mtDNA) restriction site variation (RFLP), mtDNA control region sequences, randomly amplified polymorphic DNA (RAPD) markers, and morphological characters. Individuals from the Virgin River drainage and Black Canyon represented a single RFLP haplotype and were identical for nucleotides along a portion of control region sequence. Evaluations of RAPD data demonstrated high levels of similarity among individuals and populations from this region. Leopard frogs from the Virgin River drainage and Black Canyon differed from *R. yavapaiensis* from west-central Arizona and northern Mexico in maximum parsimony and distance analyses of RFLP and control region sequence data and in maximum-likelihood analysis of the sequence data. Multidimensional scaling of RAPD data provided a similar and congruent indication of this separation. Analysis of principal component scores demonstrated significant morphological differentiation between leopard frog specimens from the Virgin River drainage and *R. yavapaiensis*. Parallel patterns of divergence observed in the mtDNA, RAPD, and morphological analyses indicate that leopard frogs from the Virgin River drainage and adjacent portions of the Colorado River are phylogenetically distinct. These leopard frogs should be recognized as a lineage separate from southern populations of *R. yavapaiensis* and classified as the species *R. onca*.

CONTROVERSY over the taxonomic validity and evolutionary distinctiveness of leopard frog species (*Rana pipiens* complex) in southern Nevada, southwestern Utah, and northwestern Arizona has confounded efforts to understand the conservation implications of the loss and decline of populations within this region. *Rana onca* Cope, the relict leopard frog, was described in 1875 from a single adult female likely collected along the Virgin River in Washington County, Utah (Cope, 1875, in Tanner, 1929). Several years later, *Rana fisheri* Stejneger, the Vegas Valley leopard frog, was described from a series of specimens collected from springs within the Las Vegas Valley, Nevada (Stejneger, 1893). The taxonomic relationship between these two nominal species has been a source of contention. Many authors considered *R. fisheri* and *R. onca* as synonyms (for citations, see Jennings, 1988), but actual comparisons between the two taxa were few and suffered from a perceived paucity of *R. onca* specimens (Slevin, 1928; Pace, 1974). Other authors, however, clearly thought the synonymy was not warranted (Linsdale, 1940; Wright and Wright, 1949; Stebbins, 1951).

Populations of *R. fisheri* within the Las Vegas Valley are thought to have gone extinct in the late 1940s as a result of habitat alterations (Stebbins, 1951). *Rana onca* populations along the Virgin River drainage were thought to have gone extinct sometime after 1950 (Jennings, 1988; J. E. Platz, Status report for *Rana onca* Cope, U.S. Fish and Wildlife Service, 1984, unpubl.). Leopard frogs persist at sites along the Virgin River from Littlefield, Arizona, downstream to areas in the Black Canyon along the Colorado River (Fig. 1), and furthering the taxonomic confusion, some of these populations (e.g., Littlefield, AZ) were more recently considered disjunct populations of *Rana yavapaiensis* Platz and Frost, the lowland leopard frog (Platz and Frost, 1984). *Rana yavapaiensis* exhibits a relatively continuous distribution extending from Sonora, Mexico, into southern and central Arizona and southwestern New Mexico, with additional populations (now thought to be extinct) in a region centered around the Imperial Valley of southern California (Fig. 1; Platz and Frost, 1984; Platz, 1988; Jennings and Hayes, 1994). *Rana pipiens* Schreber, the northern leopard frog (Schreber, 1782, in Pace, 1974), also occurs within the upper reaches of the Virgin River, but this species is extralimital to this study.

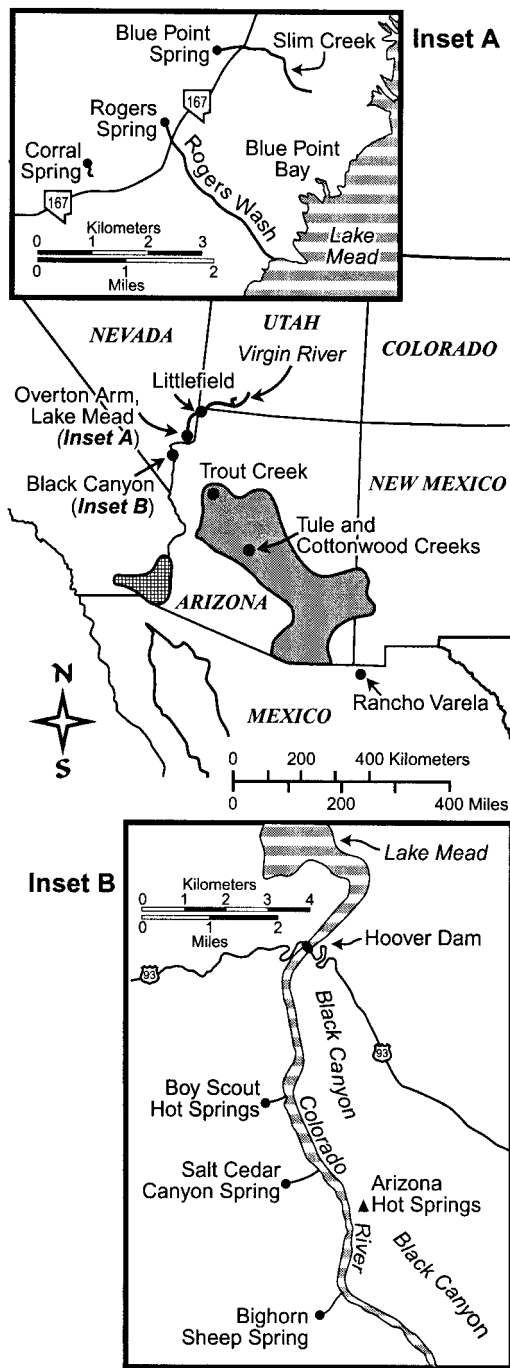Leopard frogs from the extant populations

Fig. 1. Sample sites for genetic analyses (solid circles). Shaded area refers to the mostly contiguous distribution of *Rana yavapaiensis* in the United States (after Platz and Frost, 1988); northern distributional limit in Arizona from observations (M. J. Sredl, pers. comm.). Hatched area refers to a historic distribution of putative *R. yavapaiensis* populations centered around the Imperial Valley of California, now possibly extinct (after Platz, 1988).

along the Virgin River drainage are morphologically variable, ranging from those that match the description and appearance of the type specimen of *R. onca* to those that more closely resemble *R. yavapaiensis* from southern localities (see morphological analyses below). One hypothesis to explain the presence of extant, morphologically variable leopard frogs in the Virgin River drainage is that two leopard frog taxa, and perhaps their hybrids, currently occur within the lower portions of the drainage. Alternatively, leopard frogs within this region may represent a single, morphologically variable taxon. In this paper, we use molecular and morphological evidence to evaluate these hypotheses and therefore determine the evolutionary distinctiveness of Virgin River populations with respect to *R. yavapaiensis* populations to the south. The question of the identity of extinct leopard frogs from the Las Vegas Valley (i.e., *R. fisheri*) will be dealt with elsewhere. We conclude with a consideration of the taxonomy of leopard frogs from the Virgin River drainage and adjacent portions of the Colorado River drainage in light of our genetic and morphological analyses.

MATERIALS AND METHODS

Samples.—Extensive surveys revealed seven sites in three general areas of the Virgin River drainage and adjacent portions of the Colorado River that currently contain leopard frogs: Littlefield, Arizona; Overton Arm of Lake Mead; and Black Canyon along the Colorado River (Fig. 1; henceforth referred to as "Virgin River/Black Canyon"). All seven sites are perennial spring-fed habitats. Collections for genetic analyses were made at all sites during the 1990s, but no frogs have been found at Corral Spring (Fig. 1) since 1995. Leopard frogs representing *R. yavapaiensis* from the more southern contiguous range were collected from three locations in west-central Arizona and one site in northern Mexico (Fig. 1; henceforth, all samples from Trout Creek south into Mexico will be referred to as "*R. yavapaiensis*"). Other southwestern ranid species (*Rana berlandieri, Rana blairi, Rana chiricahuensis,* and *Rana pipiens*) were included in the mtDNA analyses (see Materials Examined for collection localities). Muscle, heart, or liver tissue of sacrificed adult animals or toe tips from animals captured and then released were used to isolate genomic DNA in phenol-chloroform-isoamyl alcohol extractions.

Samples for morphological analyses were museum specimens of adult leopard frogs housed in eight regional and national collections (see

Materials Examined). Examined specimens included 53 leopard frogs from the Virgin River drainage west of Hurricane, Utah, downstream to the Overton Arm of Lake Mead in Nevada (including the type specimen of *R. onca*); five leopard frogs from the Imperial Valley of California; and 25 *R. yavapaiensis.* To better interpret morphological variation in visual analyses, specimens from the Virgin River drainage were further assigned geographical designations: Overton Arm of Lake Mead or Virgin River drainage upstream of Lake Mead. Most of the samples from sites along the Overton Arm of Lake Mead were collections from extant populations. We also, a priori, assigned a designation to those specimens from upstream locations that upon visual inspection matched the description and appearance of the type specimen of *R. onca.*

Mitochondrial DNA restriction site variation.—A total of 50 southwestern leopard frog specimens representing six nominate species were assayed for mtDNA restriction-site variation (restriction fragment-length polymorphisms; RFLP). Of these samples, 19 animals were from six of the Virgin River/Black Canyon sites and 11 animals were from three sites within the southern range of *R. yavapaiensis.* Oligonucleotide primers (Riddle et al., 1993) located in the met-tRNA (L3880) and cytochrome oxidase subunit 1 (COI) genes (H6033) were used in polymerase chain reaction (PCR) to amplify a fragment approximately 2150 basepairs (bp) in size that included the NADH subunit 2 (ND2) gene (about 1035 bp), five intervening tRNAs, and about 705 bp of the COI gene.

Eleven tetra- or heptanucleotide restriction enzymes were used in the final analysis (*Bsp*1286I, *Bst*UI, *Dpn*II, *Hae*III, *Hha*I, *Hinc*II, *Hinf*I, *Msp*I, *Rsa*I, *Sau*96IA, and a *Taq*I). Each restriction digestion was conducted using around 7µL of PCR product according to manufacturers' protocols (New England Biolabs, Inc.). Digests were electrophoresed through a 2.0% agarose gel and visualized using ethidium bromide staining. Digital photographs of each gel were made for analyses, and restriction fragment sizes were estimated by visual comparisons against molecular-weight markers run on each gel. Restriction-site gains and losses were inferred for each enzyme through direct examination of fragment patterns under the assumption that comigrating fragments from different specimens represented identical stretches of mtDNA and that fragment patterns that differed minimally by presence or absence of two fragments could be attributed to at least one restriction-site gain or loss.

Phylogenetic analyses were performed using PAUP* (Phylogenetic Analysis Using Parsimony and Other Methods, vers. 4.0b, D. L. Swofford, Sinauer Associates, Sunderland, MA. 1998, unpubl.). *Rana chiricahuensis* was selected as an outgroup based on prior evidence of a distant relationship between this species and all others examined herein (Hillis, 1988). Neighbor-joining (NJ) trees (Saitou and Nei, 1987) were constructed using a matrix of sequence divergence estimates among haplotypes (Nei and Li, 1979). Two separate maximum-parsimony (MP) analyses were conducted under different character weighting assumptions: site gains and losses weighted equally (Wagner parsimony; Farris, 1970); and site gains constrained to occur only once while multiple losses were allowed (Dollo parsimony; DeBry and Slade, 1985). Nonparametric bootstrap values were generated as a depiction of the robustness of clades on NJ and MP trees (Felsenstein, 1985; Hillis and Bull, 1993). Wilcoxon signed-rank tests (Templeton, 1983) were used to evaluate null hypotheses of no difference ($P < 0.05$) between the best MP tree and less parsimonious user-input alternative trees.

Control region sequence variation.—Nine leopard frogs from the seven Virgin River/Black Canyon sites, six leopard frogs representing *R. yavapaiensis* from four sites in west-central Arizona and northern Mexico, and representative samples of *R. berlandieri, R. blairi, R. chiricahuensis,* and *R. pipiens* were sequenced for a portion of the mtDNA control region. Primers CytbA-L and ControlP-H from Goebel et al. (1999) were used to PCR-amplify and sequence a segment of the mtDNA that was generally either 1137 bp or 1224 bp long in *R. yavapaiensis, R. blairi, R. berlandieri,* and Virgin River/Black Canyon samples, depending on the number of repeat elements and insertion/deletions. Sequences were considerably longer in *R. chiricahuensis* and *R. pipiens* (see below). Two additional sequencing primers were designed within the control region: Hrana-1232 (TCT GCG TGA TCT AAT GCA AG) was used to sequence the light-strand spanning the gap between sequences from CytbA-L and ControlP-H; and HranaA-L (GTG TAG ATA TTR AGA TGG GTA TC) was used to sequence a strand mostly complementary to that from CytbA-L.

Sequences were determined using an ABI 310 Prism automated sequencer and Big Dye Terminator Cycle Sequencing chemistry (PE Applied Biosystems, Inc.). Chromatograms were evaluated and corrected by eye. In addition to the control region sequence, raw sequences

contained about 103–106 bp of cytochrome *b* (cyt *b*). This region was aligned with published *R. catesbeiana* sequence (Yoneyama, 1987). Virgin River/Black Canyon and *R. yavapaiensis* sequences contained one additional amino acid at the three-prime end prior to the terminator. Relatively low rates of nucleotide substitution in the cyt *b* gene appear to be common in amphibians (Graybeal, 1993; Caccone et al., 1997), but the control region contains many sites that are noncoding and evolve quickly (see Taberlet, 1996). Because of the difference in evolution between these mtDNA regions, cyt *b* sequences and terminators were excluded from analyses.

In all samples, the beginning of the control region consisted of a recurrent element in various forms. In its shortest form (*R. berlandieri, R. blairi,* and most *R. yavapaiensis* and Virgin River/Black Canyon samples), the recurrent element consisted generally of 87 bp, with some sequence variation, repeated twice with a portion of the recurrent element repeated again downstream. Some *R. yavapaiensis* and Virgin River/Black Canyon sequences contained an additional version of the repeat element, and the beginning of the control region in both the *R. chiricahuensis* and *R. pipiens* consisted of longer segments that appeared to contain portions of the recurrent element. Final alignments of control region sequences were constructed using ClustalW as implemented in BioEdit (vers. 4.8.7, T. A. Hall, 1999, unpubl.). To allow meaningful comparisons of sequence data, additional repeat elements within some of the *R. yavapaiensis* and Virgin River/Black Canyon samples, along with 399 bp and 236 bp at the beginning of the *R. chiricahuensis* and *R. pipiens* sequences, respectively, were discarded prior to analyses. In this form, control region sequences included in the analysis ranged from 948 (*R. chiricahuensis*) to 962 bp with most sequences being 959 bp in size.

Maximum-likelihood (ML), MP, and NJ analyses were used to evaluate patterns of control region sequence divergence. All analyses were conducted using PAUP* with gaps ignored and *R. chiricahuensis* designated as an outgroup. An NJ tree (Saitou and Nei, 1987) was first constructed using the Tamura-Nei (1993) model of nucleotide substitution. Maximum-parsimony trees were generated using the branch-and-bound algorithm under equal weighting of all character changes. Bootstrap values were calculated to evaluate support for various clades within NJ and MP trees. Sequence data were evaluated using Modeltest (Posada and Crandall, 1998) to choose an appropriate model of sequence evolution for use in a heuristic search using the ML criterion. Modeltest uses a hierarchical approach and likelihood ratio tests to evaluate the best fit of data to a series of DNA substitution models.

RAPD variation.—Randomly amplified polymorphic DNA (RAPD) data were generated following the general outline of Williams et al. (1990). Because RAPD-PCR is sensitive to variation in reaction conditions, only samples from which DNA was recently extracted from toe tips were used in analyses. We included 102 samples from six Virgin River/Black Canyon sites and 19 *R. yavapaiensis* from two locations.

RAPD-PCR was performed in 25 μL reactions containing nuclease-free water, 3 mM MgCl$_2$, 10 mM Tris-HCl, 10 mM KCl, 0.2 mM of each dNTP, 1μM of a single 10 bp oligonucleotide primer, 1.25 unit of Taq DNA polymerase Stoffel Fragment (PE Applied Biosystems, Inc.), and approximately 30 ng of DNA. Thermal cycling consisted of an initial denaturing step of 94 C for 2.5 min, followed by 44 repetitions of 94 C for 1 min, 36 C for 1 min, 72 C for 2 min. The 72 C extension was held for 10 additional minutes during the final cycle prior to being held at 4 C. Amplification products were visualized on 2% agarose gels run for approximately 4.3 h at 93 volts and detected with ethidium bromide under UV light. Digital images of the gels were taken and printouts of these images were used for fragment scoring. Size standards (usually 100 bp ladder) were run on each gel and used to estimate fragment sizes.

A preliminary screening of 134 primers was conducted with a subsample of five or six leopard frogs representing geographically distinct sites. Primers were from random 10-bp primer kits (Operon Technologies, Inc.) or were identified as useful in a previous RAPD analysis of *R. pipiens* (Kimberling et al., 1996). Further analyses were conducted with primers that produced relatively unambiguous fragments and had at least one polymorphic fragment (marker) between any of the samples.

Markers were identified by primer name and fragment size, and each marker was scored as present (1) or absent (0). Over the course of days necessary to collect data using a particular primer, various samples were subject to repeated PCRs to confirm that markers were consistent (average = 18 samples/primer). Any marker not consistently scored between these successive PCRs was discarded; this resulted in some primers being discarded from further analysis. Only polymorphic markers showing repeatable, high-intensity amplifications were scored. This conservative approach to marker selection re-

TABLE 1. PRIMERS AND POLYMORPHIC MARKERS USED IN RAPD ANALYSES.

Primer	Sequence	Band Scored (base pairs)
OPA-07	GAAACGGGTG	740
OPA-07		460
OPA-08	GTGACGTAGG	390
OPA-11	CAATCGCCGT	550
OPA-11		410
OPA-11		290
OPA-12	TCGGCGATAG	660
OPA-12		610
OPA-12		425
OPA-12		400
OPA-19	CAACGTCGG	600
OPA-19		525
OPA-19		500
OPI-05	TGTTCCACGG	290
OPI-07	CAGCGACAAG	400
OPJ-09	TGAGCCTCAC	710
OPJ-09		400
UBC-42	TTAACCCGGC	250
UBC-42		235
UBC-217	ACAGGTAGAC	440
UBC-217		420

sulted in the scoring of 10 primers for 21 markers (Table 1).

Similarity between two samples was calculated using the Dice formula (Nei and Li, 1979) as implemented by the program NTSYSpc (Numerical Taxonomy and Multivariate Analysis System, vers. 2.0, F. J. Rohlf, Exeter Software, Setauket, NY, 1998, unpubl.). Index values for this measure range from 1 (identical) to 0 (no similarity). Similarity between two sample sites was calculated using the formula:

$$\bar{S}_{ij} = 1 + \bar{S}_{ij}' - \frac{\bar{S}i + \bar{S}j}{2}$$

where $\bar{S}i$ and $\bar{S}j$ are the average similarity between individuals within sample sites i and j, respectively, and $\bar{S}ij'$ is the average similarity between individuals among sample sites i and j (Wright, 1965; Liao and Hsiao 1998). Using this formula, the index of intersite similarity is corrected by intrasite similarities. Multidimensional scaling (MDS) analyses based on the pairwise similarity matrixes were performed as implemented in NTSYSpc. Because problems with multiple local minima is possible in MDS, principle coordinates analysis (PCoA) was first conducted on each dataset and used as the initial configuration matrix for the respective MDS analysis. Using the results from a PCoA as an initial configuration matrix assures that the results from MDS will not be worse than that of a

PCoA analysis (NTSYSpc, F. J. Rohlf, 1998, unpubl.).

Morphological analyses.—Eight continuous morphological traits were measured, and 11 discontinuous traits were coded on each museum specimen. Coding protocols for discrete characters were developed to create an ordinal series of character states (for coding protocols, see Appendix 1). Continuous traits were head width, head length, lip height, internarial distance, tympanum diameter, eye diameter, tibiofibula length, and snout–urostyle length. Discontinuous traits were the condition of the dorsolateral folds, number of spots anterior to the eyes, number of spots on the head above the eyes, number of dorsal spots between the dorsolateral folds, number of bars on the dorsal surface of the thigh, condition of the thigh pattern, condition of the tympanum spot, degree of the mottling on the lower lip, degree of mottling on the chin, condition of the supralabial stripe, and extent of the webbing on the hind foot.

The 19 morphologic traits were subjected to principal components analysis (PROC FACTOR; SAS vers. 6, Statistical Analysis Systems Institute, Inc., Cary, NC, 1985, unpubl.) to understand better the relationships among variables and to identify major trends in the data. Continuous and discontinuous data were ranked prior to analysis to reduce nonnormality (Conover, 1980; Conover and Iman, 1981) and to allow both types of data to be used in the same analysis. Principal components satisfying a minimum eigenvalue-equal-one criterion were plotted against each other using species/geographical designations as markers to elucidate major groups of leopard frogs (cluster analysis). Initially, cluster plots were performed separately for each sex to eliminate conflicting patterns between the sexes. Principal component scores for individual frogs were subjected to a two-way analysis of variance with interaction using major species/geographical designation (all samples from the Virgin River drainage were assigned a single geographical designation) and sex as class variables (PROC GLM; SAS vers. 6, unpubl.) followed by pairwise comparisons of least-squared means.

RESULTS

Restriction site variation.—From assay of restriction site variation, 12 composite mtDNA haplotypes were inferred among 50 individuals representing six nominate species (for haplotypes and restriction site variation data, see Appendix

2). Presence or absence of distinct restriction sites could generally be inferred through examination of variable fragment patterns. This approach could not be employed to deduce homologous restriction sites between *R. chiricahuensis* and other species for three restriction enzymes because of increased RFLP pattern complexity at this level of divergence. These characters were therefore coded as ''missing information'' in *R. chiricahuensis*. A total of 60 restriction sites were thus recorded, 40 being variable excluding *R. chiricahuensis*. All individuals ($n = 19$) from the Virgin River/Black Canyon sites possessed a single haplotype not found elsewhere (01 Virgin River/Black Canyon). Five haplotypes were found in 11 individuals from the three southern populations of *R. yavapaiensis* (02–06 *R. yavapaiensis*). None of the *R. yavapaiensis* haplotypes was shared with other species of leopard frogs. Six additional haplotypes were distributed among *R. blairi, R. berlandieri, R. chiricahuensis,* and *R. pipiens*. Pairwise estimates of sequence divergence among haplotypes (excluding *R. chiricahuensis*) ranged from low values of 0.2% (between haplotypes 09 and 10) and 0.3% (haplotypes 03,04; 03,05; 05,06) to high values of about 10–11% between *R. pipiens* haplotypes 09 and 10 and any of the *R. yavapaiensis* and Virgin River/Black Canyon haplotypes (Table 2).

Parsimony analysis performed under Wagner and Dollo criteria produced MP trees (Fig. 2A) that were identical in topology for major clades (Wagner: length = 57, CI = 0.77, RI = 0.88; Dollo: length = 60, CI = 0.73; RI = 0.94). These trees indicated a monophyletic clade uniting haplotypes 02–06 *R. yavapaiensis* with haplotype 01 Virgin River/Black Canyon, a separate *R. berlandieri* + *R. blairi* clade, and a basal *R. pipiens* clade (Fig. 2A). Both Wagner and Dollo MP trees indicated a clade consisting of *R. yavapaiensis* haplotypes 02–06 relative to the Virgin River/Black Canyon haplotype 01, but this relationship received stronger bootstrap support under Dollo parsimony (Bootstrap = 82). Statistical evaluations (one-tailed Wilcoxon signed-rank tests) of the MP tree against user-input alternative trees that joined haplotypes 01 Virgin River/Black Canyon and 02 *R. yavapaiensis* or 06 *R. yavapaiensis* into a clade indicated that the alternative trees were significantly worse under both Wagner (haplotypes 01 with 02: length = 59, $P = 0.03$; haplotypes 01 with 06: length = 59, $P = 0.03$) and Dollo (haplotypes 01 with 02: length = 63, $P = 0.04$; haplotypes 01 with 06: length = 63, $P = 0.04$) parsimony. If the user-input tree further eroded the *R. yavapaiensis* + Virgin River/Black Canyon

TABLE 2. MATRIX OF SEQUENCE DIVERGENCE ESTIMATES (NEI AND LI, 1979) AMONG HAPLOTYPES DETERMINED FROM RESTRICTION SITE VARIATION DATA. Haplotype 01 references the identical haplotype found in 19 leopard frogs representing six sites from the Virgin River drainage and Black Canyon.

| Haplotype no. and taxon	Haplotype number											
	01	02	03	04	05	06	07	08	09	10	11	12
01 Virgin R./Black C.	—											
02 *R. yavapaiensis*	0.0170	—										
03 *R. yavapaiensis*	0.0204	0.0090	—									
04 *R. yavapaiensis*	0.0239	0.0124	0.0031	—								
05 *R. yavapaiensis*	0.0170	0.0059	0.0030	0.0061	—							
06 *R. yavapaiensis*	0.0204	0.0090	0.0061	0.0094	0.0030	—						
07 *R. berlandieri*	0.0732	0.0625	0.0680	0.0651	0.0708	0.0766	—					
08 *R. berlandieri*	0.0732	0.0708	0.0766	0.0738	0.0794	0.0766	0.0164	—				
09 *R. pipiens*	0.0978	0.0967	0.1140	0.1113	0.1064	0.1037	0.0705	0.0784	—			
10 *R. pipiens*	0.0953	0.0940	0.1113	0.1085	0.1037	0.1010	0.0758	0.0841	0.0024	—		
11 *R. blairi*	0.0628	0.0598	0.0738	0.0709	0.0680	0.0651	0.0138	0.0138	0.0605	0.0654	—	
12 *R. chiricahuensis*	0.0940	0.0967	0.1113	0.1057	0.0967	0.0913	0.1057	0.0913	0.1113	0.1057	0.0680	—
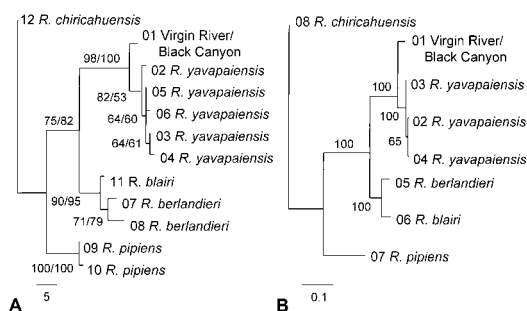
Fig. 2. (A) Maximum parsimony tree under Dollo criteria. *Rana chiricahuensis* was selected as the outgroup. Virgin River/Black Canyon references a single haplotype found in 19 leopard frogs from six sites in the Virgin River drainage and Black Canyon. Bootstrap support is indicated for major clades under both Dollo (first score) and Wagner (second score) parsimony. (B) Maximum-likelihood tree generated from control region sequence data under the HKY85 model (Hasegawa et al., 1985). Virgin River/Black Canyon references a single haplotype found in nine leopard frogs from the seven sites in the Virgin River drainge and Black Canyon. *Rana chiricahuensis* was selected as the outgroup. Bootstrap support is indicated for the same tree generated by maximum parsimony with character state changes unweighted.

clade, by joining haplotype 01 with the *R. berlandieri* + *R. blairi* clade, the alternative tree was significantly worse than the original MP tree under both Wagner (length = 67, $P = 0.0008$) and Dollo (length = 72, $P = 0.00025$) parsimony.

A NJ tree (not shown) constructed using the distance matrix (Table 2) was consistent with MP trees in indicating a monophyletic clade uniting haplotypes 02–06 *R. yavapaiensis* with haplotype 01 Virgin River/Black Canyon. This clade was established in 100% of the bootstrap replicates. A clade consisting of *R. yavapaiensis* haplotypes 02–06 relative to the haplotype 01 Virgin River/Black Canyon was supported, but only weakly (bootstraps = 68%). The NJ tree differed from MP trees by weakly supporting a clade consisting of *R. berlandieri, R. blairi,* and *R. pipiens* (bootstraps < 50%).

Control region sequence variation.—Sequence data consisted of eight haplotypes (including the outgroup). Control region sequence nucleotides were identical for all nine individuals from the seven Virgin River/Black Canyon sites (haplotype 01; Table 3). Three haplotypes were identified from the six individuals representing *R. yavapaiensis* populations with haplotypes differing from each other by Tamura-Nei distances of 0.0031–0.0052 (haplotypes 02–04; Table 3). The Virgin River/Black Canyon haplotype differed

TABLE 3. MATRIX OF TAMURA-NEI (1993) DISTANCES FROM CONTROL REGION SEQUENCES. Haplotype 01 references the identical sequences from nine individuals representing the seven sample sites within the Virgin River drainage and Black Canyon. Site names for *Rana yavapaiensis* haplotypes reference Figure 1. Sample information is listed in the text under Materials Examined. Representative control region sequences are referenced in GenBank by accession numbers AF343776–AF343783.

Haplotype no. and taxon	Samples	Haplotype number							
		01	02	03	04	05	06	07	08
01 Virgin R./Black Cyn.	All samples	—							
02 *R. yavapaiensis*	Trout: LVT 4560 Cottonwood: LVT 4566 Tule: LVT 4575	0.0468	—						
03 *R. yavapaiensis*	Trout: LVT 4561 Rancho Varela: NK 3930	0.0456	0.0031	—					
04 *R. yavapaiensis*	Trout: LVT 4562	0.0490	0.0042	0.0052	—				
05 *R. blairi*	RDJ 899	0.1432	0.1475	0.1461	0.1474	—			
06 *R. berlandieri*	LVT 4594	0.1404	0.1427	0.1413	0.1426	0.0515	—		
07 *R. pipiens*	LVT 4583	0.2539	0.2604	0.2603	0.2651	0.2471	0.2496	—	
08 *R. chiricahuensis*	NK 3318	0.2462	0.2567	0.2565	0.2563	0.2364	0.2301	0.1895	—

TABLE 4. PAIRWISE SIMILARITY MATRIX BASED ON RAPD DATA. The column labled "Within" provides the average pairwise similarity between samples (Nei and Li, 1979) within a sample site and sample size in parentheses. Following columns present similarity values among sample sites after correcting for average within-site similarities. Sites 1 through 6 are Virgin River and Black Canyon locations, 7 and 8 are *Rana yavapaiensis* sites from west-central Arizona. Site names reference Figure 1. Sample information is listed in the text under Materials Examined.

Site no. and location	Within (n)	Site number							
		1	2	3	4	5	6	7	8
1 Littlefield	0.9235 (13)	—							
2 Blue Point	0.9929 (29)	0.9177	—						
3 Rogers	0.9559 (4)	0.9388	0.9978	—					
4 Boy Scout	0.9870 (21)	0.9777	0.9430	0.9598	—				
5 Salt Cedar	0.9638 (7)	0.9476	0.9984	1.0008	0.9726	—			
6 Bighorn	0.9898 (28)	0.9433	0.9704	0.9644	0.9761	0.9865	—		
7 Trout	0.7295 (8)	0.5267	0.4813	0.4990	0.5123	0.5034	0.4959	—	
8 Cottonwood	0.8433 (10)	0.5703	0.5149	0.5348	0.5504	0.5383	0.5317	0.9379	—

from sequences of *R. yavapaiensis* by 42–45 nucleotide changes which resulted in Tamura-Nei distances of 0.0456–0.049 (Table 3). All other haplotypes were representative of the species included in the analyses.

Evaluation of the sequence data using Modeltest indicated that the HKY85 (Hasegawa et al., 1985) model with gamma distributed rate heterogeneity was an appropriate model of nucleotide substitution. Using this model, a heuristic search (random addition, one replication, tbr branch swapping) recovered an ML tree ($-$lnL score = 3189.17428, α = 0.449607) with a topology nearly identical to that produced from RFLP MP analyses (Fig. 2B). Neighbor-joining and MP analyses both produced trees with nearly identical topologies to the ML tree. Support was strong (Bootstraps = 100%) in both the NJ and MP analyses for major clades and for a monophyletic relationship between *R. yavapaiensis* haplotypes (02–04) and the Virgin River/Black Canyon haplotype (01).

RAPD variation.—Estimates of average within-population similarity between pairs of individuals derived from RAPD data were high for Virgin River/Black Canyon sites (sites 1–6; Table 4). Among-population similarity within this region was also high (similarity between any pair of Virgin River/Black Canyon sites was $\geq$ 0.918; Table 4). The two *R. yavapaiensis* populations (sites 7–8; Table 4) showed somewhat lower average similarity for pairs of samples within each population, but the similarity between these two populations was high (similarity = 0.938). Similarity values decreased markedly in contrasts between Virgin River/Black Canyon sites and the two *R. yavapaiensis* populations (average similarity between pairs of Virgin River/Black

Canyon sites and *R. yavapaiensis* sites ranged from 0.481–0.570; Table 4).

The MDS analysis based on pairwise similarity between individuals demonstrated the divergence between Virgin River/Black Canyon frogs and *R. yavapaiensis* using two dimensions (Fig. 3A). A measure of the goodness-of-fit between MDS results (distances in the configuration space) and the monotone function of the original distances was good (Stress 2 = 0.068; NTSYSpc, F. J. Rohlf, 1998, unpubl.). A complementary pattern was obtained from the MDS analysis based on the pairwise similarity between sample sites (Stress 2 = 0.008; Fig. 3B). Further interpretation of the RAPD data to derive estimates of the relationships among Virgin River/Black Canyon sample sites was not considered useful. Of the 21 RAPD markers used in this analysis, only seven markers showed any variability within and among these populations, and the Littlefield individuals manifested the majority of this variation. Regardless of this variation, the Littlefield individuals and population clearly grouped with other individuals and sites from the Virgin River drainage and Black Canyon (Fig. 3).

Morphological analyses.—Principal components analyses of morphological data conducted for each of the sexes were markedly consistent in content; therefore, subsequent results of principal components analyses are with the sexes combined. Five principal components (PCs) that possessed eigenvalues greater than one, explained 74.2% of the overall morphological variance (Table 5). The first principal component, accounting for 38.6% of the total variation, had positive loading on all linear measurements and, therefore, represents overall size. Bivariate
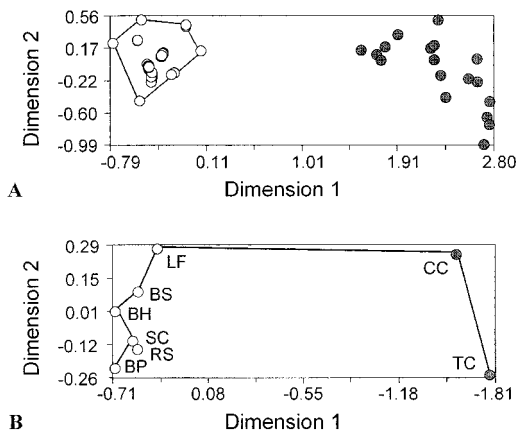
Fig. 3. (A) Multidimensional scaling (MDS) plot based on pairwise similarity values between individual leopard frogs derived from RAPD data. Individuals from the Virgin River drainage and Black Canyon are indicated by open circles. The polygon encloses all 102 samples from this region. Individuals from west-central Arizona populations of *Rana yavapaiensis* are indicated by dark circles. (B) MDS plot based on pairwise similarity values between sample sites, with a minimum spanning network superimposed. Sample sites reference Figure 1 (Littlefield = LF, Blue Point = BP, Rogers = RS, Boy Scout = BS, Salt Cedar = SC, Bighorn = BH, Trout Creek = TC, Cottonwood Creek = CC). Dimension 1 of graph B has been multiplied by −1 for display purposes (only the relative positions of objects are important in MDS configurations and relative position is unchanged by reflection of scales).

plots of principal components were used to visualize patterns among the designated groups of leopard frogs. The plot of PC II against PC V (Fig. 4) exhibited the greatest discriminatory power among groups of leopard frogs and elucidated a difference between leopard frogs from the Virgin River drainage (no *R. pipiens* from upstream locations were included in the analysis) and *R. yavapaiensis* specimens. Principal component II explained 14.9% of the overall variance. Number of thigh bars, number of dorsal spots, number of spots between the eyes, number of spots anterior to eyes, condition of the supralabial stripe, condition of the tympanum spot, amount of chin mottling, and thigh pattern loaded heavily and positively on PC II. PC V explained just 6.0% of the overall variance but tended to separate leopard frogs from the Imperial Valley from the other groups. Number of dorsal spots, number of eye spots, extent of webbing, and condition of dorsolateral folds loaded positively, whereas condition of the tympanum spot, condition of the thigh pattern, and condition of the supralabial stripe loaded negatively on PC V.

A minimum convex polygon encompassing all leopard frogs from the Virgin River drainage included only two of the 25 *R. yavapaiensis* specimens (Fig. 4). Specimens considered consistent with the description and appearance of the type specimen of *R. onca*, occupied a subset of the

TABLE 5. MORPHOLOGICAL VARIABLES USED IN PRINCIPAL COMPONENTS ANALYSIS AND THEIR EIGENVECTOR LOADINGS AMONG MAJOR PRINCIPAL COMPONENTS (EIGENVECTOR LOADINGS WITH A MAGNITUDE LESS THAN |0.20| ARE NOT SHOWN).

| Variable	PC I	PC II	PC III	PC IV	PC V
Head width	0.36				
Head length	0.34				
Lip height	0.35				
Internarial distance	0.32				
Tibiofibula length	0.35				
Snout–urostyle length	0.36				
Eye diameter	0.30				
Tympanum diameter	0.32				
Dorsolateral folds			0.64		0.24
Nose spots		0.40			
Eye spots		0.40			0.31
Dorsal spots		0.44			0.34
Thigh bars		0.47	0.21		
Thigh pattern		0.21	0.42		−0.32
Tympanum spot		0.23			−0.63
Lower lip mottling			0.27	0.52	
Chin mottling		0.22	−0.21	0.56	
Supralabial stripe		0.32	−0.35		−0.29
Webbing				0.54	0.30
Proportion of variance	38.6%	14.9%	7.7%	7.0%	6.0%
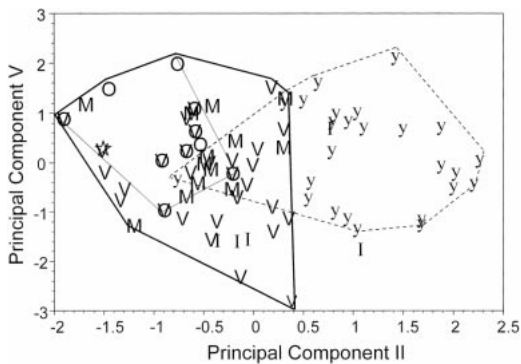
Fig. 4.   A plot of principal component scores for PC II and PC V, derived from morphological data, by species or geographical designation (M = specimens from the Overton Arm of Lake Mead, V = specimens from the Virgin River drainage upstream of Lake Mead, O = specimens from the Virgin River drainage upstream of Lake Mead that matched the appearance of *Rana onca*, I = specimens from the Imperial Valley, y = *Rana yavapaiensis* from the main distribution in Arizona). The type specimen of *R. onca* is indicated by a star. Lines describe minimum convex polygons for all specimens from the Virgin River drainage (heavy solid line), frogs consistent with the appearance and description of *R. onca* (light solid line), and for *R. yavapaiensis* specimens from western Arizona (broken line). Specimens considered to match the type description and appearance of *R. onca* consisted of the following: BYU 9686, BYU 9691–9692, BYU 9702–9703, BYU 12766–12768, and LACM 106083–106084.

morphological variability exhibited by leopard frogs from throughout the Virgin River drainage; but more important, many leopard frogs from the Overton Arm of Lake Mead and from upstream locations along the Virgin River drainage were similar on bivariate plots to those we considered consistent with *R. onca*. Indeed, although the *R. onca* type specimen scored lower on PC II, several specimens from both the Overton Arm of Lake Mead and from upstream locations had very similar scores. Leopard frogs from the Imperial Valley generally appeared to be intermediate between frogs from the Virgin River drainage and *R. yavapaiensis*.

Two-way analysis of variance (with interaction) using major species/geographical designation (i.e., all frogs from Virgin River drainage combined, Imperial Valley frogs, and *R. yavapaiensis*) and sex as class variables were conducted for the first five principal components. Interaction terms for all analyses were not significant. Only PC II exhibited significance within the ANOVA model (F = 24.67, df = 5,77, P = 0.0001). Species/geographic groups were differentiated by PC II (F = 58.52, df = 2,77, P =

0.0001) with *R. yavapaiensis* differing from both Virgin River (P = 0.0074) and Imperial Valley frogs (P = 0.0001) in pairwise comparisons of least-square means. Leopard frogs from the Virgin River drainage did not differ significantly from the small sample of Imperial Valley frogs (P = 0.085). When the Imperial Valley frogs (currently considered *R. yavapaiensis*) were assigned to the *R. yavapaiensis* group, however, a significant difference between leopard frogs from the Virgin River drainage and more southern populations was retained (F = 100.86, df = 1,79, P = 0.0001).

DISCUSSION

Both mitochondrial and RAPD markers provided a clear signal of historical separation between populations of leopard frogs that occupy the Virgin River drainage and Black Canyon from populations of *R. yavapaiensis* in west-central Arizona and northern Mexico. Furthermore, each genetic marker provided evidence for either identical or very similar genotypes among populations ranging from Littlefield, Arizona, downstream to Bighorn Sheep Spring in Black Canyon (Fig. 1). Maximum-parsimony and NJ trees produced from mtDNA RFLP data generally supported a monophletic relationship between *R. yavapaiensis* haplotypes relative to the Virgin River/Black Canyon haplotype within the clade containing both groups. Sequences of mtDNA control region provided a more robust depiction of divergence between Virgin River/Black Canyon samples versus *R. yavapaiensis* in ML, MP, and NJ analyses. The difference in degree of resolution between datasets likely derives from both a higher mutation rate in the control region sequence as well as a difference in resolution between indirect (RFLP) and direct (sequencing) protocols. RAPD markers provide a similar and completely congruent indication of separation between Virgin River/Black Canyon populations and *R. yavapaiensis* populations within the total genome (reflecting variation mostly in the nuclear genome).

The hypothesis that leopard frogs from the Virgin River drainage actually represent both *R. onca* and *R. yavapaiensis* populations and possibly their hybrids is inconsistent with the genetic evidence. Congruence between mitochondrial RFLP, control region sequences, and RAPD markers in demonstrating a substantial subdivision of populations into northern (Virgin River/Black Canyon) and southern (*R. yavapaiensis*) lineages argues against the presence of two species or hybridization along the Virgin River drainage and Black Canyon.

Morphologically, leopard frogs from the Virgin River drainage and *R. yavapaiensis* populations appear to exhibit a continuum of multivariate variation with leopard frogs from the Virgin River and *R. yavapaiensis* comprising different ends of the spectrum. That individuals from these two groups of leopard frogs can appear very similar has been the source of much taxonomic confusion. Our morphological analysis, however, provides quantitative evidence that significant differences exist between the Virgin River leopard frogs and *R. yavapaiensis*. Although the sample size from the Imperial Valley was too small for conclusive analysis, the relationship between the Imperial Valley populations and Virgin River/Black Canyon populations may reflect a common ancestral lineage that evolved along the Colorado River from which all groups are derived. This possibility is supported by the basal position of the Virgin River/Black Canyon leopard frogs on mtDNA trees relative to *R. yavapaiensis* populations (Fig. 2). When the specimens from the Imperial Valley were forced into the *R. yavapaiensis* group in morphological analyses, however, leopard frogs from the Virgin River drainage remained significantly different from more southern populations.

The concept of Evolutionarily Significant Units (ESUs) provides an objective foundation for delineating conservation units with attention to preservation of evolutionary processes (Moritz, 1994a). Under one definition, a group of populations are considered an ESU, regardless of taxonomic designation, if there is substantial evidence of long-term isolation from other populations as determined by a significant phylogenetic structuring of mitochondrial DNA and evidence of substantial divergence in nuclear DNA (Moritz, 1994a,b). The genetic patterns observed in this study provide a compelling argument for recognition of leopard frog populations from the Virgin River drainage and Black Canyon as a distinct ESU relative to populations of *R. yavapaiensis* south of Black Canyon. We argue that ESU recognition provides a sound basis for developing conservation management strategies that retain the Virgin River/Black Canyon populations as a separate lineage relative to *R. yavapaiensis*. Other than leopard frogs (purportedly *R. yavapaiensis*) in the Bill Williams River drainage (Trout Creek is in this drainage system) near the confluence with the Colorado River, we know of no extant populations along the Colorado River south of Black Canyon. We caution, however, that future sampling may show the northern genotype to be established further south along the Colorado River.

The taxonomic history of leopard frogs from the Las Vegas Valley, Virgin River drainage, and adjacent Colorado River is complex, but the genetic data presented here are sufficient to further recognize the leopard frogs from the Virgin River drainage and Black Canyon as a species distinct from more southern *R. yavapaiensis*. The morphological differences between leopard frogs from the Virgin River drainage and *R. yavapaiensis* are consistent with a species level designation between these taxa. Leopard frogs from the Virgin River drainage and Black Canyon should be recognized by the historic name *R. onca* because of the presence of individuals from both extinct and extant populations that match the description and appearance of the type specimen of *R. onca* and because genetic data allow rejection of the hypothesis that extant populations represent a current introgression of *R. yavapaiensis*.

Rana onca populations from the Virgin River/Black Canyon probably represent relatively recent (e.g., late Pleistocene-Holocene) isolates from ancestral populations further to the south. The Virgin River and Black Canyon are in the Mojave Desert and are currently peripheral to areas of the Sonoran Desert occupied by *R. yavapaiensis*. We are uncertain whether *R. onca* populations represent a northern expansion of *R. yavapaiensis*, which then became isolated as a result of habitat changes caused by fluctuating climatic conditions, or whether *R. onca* represent remnant, northern populations of a western version of these frogs that once were widely distributed along the Colorado River.

Current evidence suggests that *R. onca* has little genetic diversity within and among extant populations. Given the high level of similarity in all evaluated genetic markers, little information can be derived from our study regarding current gene flow and population structure. A higher-resolution technique might provide the sensitivity required to estimate patterns and rates of gene flow among extant populations, thereby providing a genetic basis for developing conservation strategies beyond recognition of the distributional limits of *R. onca*. Meanwhile, a conservation management plan for the few remnant *R. onca* populations should prioritize the identification of habitat requirements and the reclamation of habitats necessary to maintain population viability. Given our discovery of the extant Black Canyon populations during surveys in 1997 and 1998, it seems prudent to also recommend a more thorough survey of potential habitats within the known range of leop-

ard frogs in the region as well as into adjacent areas along the Colorado River.

MATERIALS EXAMINED

Specimens or tissue samples used in each analysis are listed by species, state, county, and site name when available. Alphabetic collection codes follow those listed in Leviton et al. (1985). NK reference samples from the University of New Mexico (MSB). LVT reference samples at the Department of Biological Sciences, University of Nevada, Las Vegas. RDJ represents uncataloged specimens. RDJ samples used in morphological analyses are voucher specimens that have not yet been accessioned at the Barrick Museum of Natural History, University of Nevada, Las Vegas. Letters following some UMMZ specimens distinguish frogs within a lot assigned a single collection number.

Restriction site variation analysis.—*Rana* spp. (*onca*): Nevada: Clark County: Blue Point Springs RDJ 916, RDJ 918; Corral Spring RDJ 921, RDJ 925, RDJ 927; Boy Scout Canyon LVT 3405, LVT 3426; Salt Cedar Canyon LVT 3411–3413; Bighorn Sheep Spring, LVT 3439–3441; Arizona: Mojave County: Littlefield RDJ 1012–1013, RDJ 1015–1017, RDJ 1022. *Rana yavapaiensis*: Arizona: Mojave County: Trout Creek RDJ 1024, RDJ 1026, RDJ 1028–1029; Yavapai County: Tule Creek RDJ 1205, RDJ 1208; Mexico: Sonora: Sierra San Luis at Rancho Varela NK 3926–3927, NK 3929-3930, NK 3933. *Rana blairi*: Arizona: Cochise County: Sulphur Springs Valley RDJ 1196, RDJ 1201; New Mexico: Sierra County: Rio Grande at Las Palomas, RDJ 908–909; San Miguel County: Conchas River RDJ 899–900; Oklahoma: Greer County: RDJ 1182, RDJ 1184. *Rana berlandiari*: Arizona: Maricopa County: North Tank (introduced) LVT 4564; Texas: Brewster County: Rio Grande Village LVT 4594. *Rana chiricahuensis*: Arizona: Cochise County: Chiricahua Mountains NK 3318–3319. *Rana pipiens*: Nevada: Lincoln County: Pahranagat Valley RDJ 1083–1084; Utah: Washington County: Green Lake RDJ 1187–1188; Fife Creek RDJ 1189; New Mexico: Rio Arriba County: El Rito NK 3948–3949; Mora County: Sierra Bonita RDJ 903.

Control region sequence analysis.—*Rana* spp. (*onca*): Nevada: Clark County: Blue Point Springs LVT 3542; Corral Spring RDJ 925; Rogers Spring LVT 4556–4557; Boy Scout Canyon LVT 3427; Salt Cedar Canyon LVT 3413; Bighorn Sheep Spring LVT 3445; Arizona: Mojave County: Littlefield LVT 3537–3538. *Rana yava-*

paiensis: Arizona: Mojave County: Trout Creek LVT 4560–4562; Yavapai County: Cottonwood Creek LVT 4566; Tule Creek LVT 4575; Mexico: Sonora: Sierra San Luis at Rancho Varela NK 3930. *Rana blairi*: New Mexico: San Miguel County: Conchas River RDJ 899. *Rana berlandiari*: Texas: Brewster County: Rio Grande Village LVT 4594. *Rana chiricahuensis*: Arizona: Cochise County: Chiricahua Mountains NK 3318. *Rana pipiens*: Nevada: Lincoln County: Pahranagat Valley LVT 4583.

RAPD analysis.—*Rana* spp. (*onca*): Nevada: Clark County: Blue Point Springs LVT 3540–3568; Rogers Spring LVT 4556–4559; Boy Scout Canyon LVT 3426–3428, LVT 3430–3438, LVT 4375–4383; Salt Cedar Canyon LVT 3411–3413, LVT 3467–3470; Bighorn Sheep Spring LVT 3439–3440, LVT 3442–3453, LVT 4502–4512, LVT 4514–4516; Arizona: Mojave County: Littlefield LVT 3500–3505, LVT 3533–3539. *Rana yavapaiensis*: Arizona: Mojave County: Trout Creek LVT 4560–4563, LVT 4578–4581; Yavapai County: Cottonwood Creek LVT 4565–4574.

Morphological analyses.—*Rana onca* (syntype): Utah: Washington County: near St. George(?) USNM 25331. *Rana* spp. (*onca*): Nevada: Clark County: Blue Point Spring RDJ 916, RDJ 918–919, 921, RDJ 929, RDJ 1063; Roger Springs CM 52423, RDJ 922; Corral Spring RDJ 923–927, RDJ 1062; Virgin River near Glendale LACM 74523. Arizona: Mohave County: Littlefield LACM 106069, RDJ 1022–1023. Utah: Washington County: Berry Spring BYU 9685–9687, BYU 9690–9692, BYU 9696, BYU 9699, BYU 9702–9703; Harrisburg Creek BYU 12766–12768; near Leeds LACM 106082–106084; near Bloomington BYU 1276, BYU 1538, LACM 91372–91373, LACM 91375; near St. George BYU 1140, BYU 2782, BYU 12769, CAS 54108–541113, CAS 54117, UMMZ 88543A–B&D. *Rana* spp. (*yavapaiensis*; Imperial Valley): California: Imperial County: LACM 13837, LACM 91311–91312; Riverside County: LACM 91310, LACM 91313. *Rana yavapaiensis*: Arizona: Maricopa County: Cave Creek CAS 17570–17573, CAS 17720, CAS 17722–17724, CAS 17727–17728, CAS 17732, CAS 17735, CAS 20856; Mohave County: near Wikieup LACM 91376; Burro Creek LACM 91377–91379; Trout Creek RDJ 1024–1027, 1029–1032.

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APPENDIX 1. DESCRIPTIONS OF CONTINUOUS MORPHOLOGICAL CHARACTERS (1–8) AND CODING PROTOCOLS FOR DISCONTINUOUS MORPHOLOGICAL CHARACTERS (9–19) USED IN MORPHOLOGICAL ANALYSES.

1. Head width: at mid tympanum on the jaw.
2. Head length: from the angle of the jaw to the tip of the snout.
3. Lip height: from the ventral margin of the upper lip to the nostril.
4. Internarial distance: from nostril to nostril.
5. Tibiofibula length: from knee to ankle gently pressing soft tissue.
6. Body length: from the urostyle to the snout.
7. Tympanum diameter: measured at its greatest diameter.
8. Eye diameter: measured at its greatest diameter.
9. Number of spots anterior to eyes: a count of dorsal spots anterior to the eyes.
10. Number of spots between eyes: a count of the spots on top of and between the eyes.
11. Number of dorsal spots: a count of spots behind the eyes and between dorsolateral folds.
12. Number of transverse bars: a count of the bars or spots on the dorsal surface of thigh.
13. Condition of the dorsolateral folds: 1 = continuous folds, 2 = broken posteriorly, 3 = broken and inset medially, 4 = not well defined posterior to break.
14. Pattern on the posterior surface of the thigh: 1 = immaculate, 2 = spotted, 3 = obscurely (fussily) reticulated, 4 = distinctively reticulated, 5 = white spots on a dark field, 6 = coalescing white spots or a white reticulation on a dark field, 7 = obscurely dark, fuscous with no discernible pattern.
15. Melanic pigment on lower lip: 1 = conspicuously mottled, 2 = obscurely mottled, 3 = flecked, 4 = suffused, 5 = immaculate.
16. Melanic pigment on the chin: character states as for melanic pigment on lower lip.
17. Condition of the tympanum spot: 1 = absent, 2 = faint or obscure, 3 = conspicuous.
18. Condition of the supralabial stripe: 1 = absent, 2 = well defined posterior to the eye, 3 = present posterior and anterior to the eye but not conspicuous, 4 = as in 3 but conspicuous.
19. Condition of webbing on fourth toe: 1 = extending onto terminal phalange, 2 = to the distal tip of subterminal phalange, 3 = about midway along the subterminal phalange, 4 = to the distal tip of third phalange, 5 = about midway along the third phalange.

APPENDIX 2. RESTRICTION SITE VARIATION DATA WITH IDENTIFIED HAPLOTYPES. Restriction sties are coded as binary data. Haplotype 01 references the identical haplotype found in 19 leopard frogs from six sites in the Virgin River drainage and Black Canyon. Site data indicated for *Rana yavapaiensis* haplotypes reference Figure 1. Sample information is listed in text under Materials Examined.

Haplotype no. and taxon designation	n	Samples	*Bsp* 12861	*Bst* UI	*Hae* III	*Hha* I	*Hinc* II	*Hinf* I	*Dpn* II	*Msp* I	*Rsa* I	*Sau* 96IA	*aTaq* I
01 Virgin R./Black C.	19	All Samples	11	110	111101110	010	10101	001000	001110000	10100101	110	11100100	1111
02 *R. yavapaiensis*	1	Trout: RDJ 1024	01	110	110101110	000	00101	001000	001110000	10000101	110	11100110	1111
03 *R. yavapaiensis*	2	Trout: RDJ 1026, 1029	01	110	111101010	000	00101	001000	001110000	10000101	110	10100110	1111
04 *R. yavapaiensis*	1	Trout: RDJ 1028	01	110	111101010	000	00101	001000	001110000	10000101	100	10100110	1111
05 *R. yavapaiensis*	2	Tule: RDJ 1205, 1208	01	110	111101010	000	00101	001000	001110000	10000101	110	11100110	1111
	2	R. Varela: NK 3929, 3933											
06 *R. yavapaiensis*	3	R. Varela: NK 3926, 3927, 3930	01	010	111101010	000	00101	001000	001110000	10000101	110	11100110	1111
07 *R. berlandieri*	1	LVT 4564	01	100	010101110	000	00101	111110	011011010	10010111	000	10111100	0111
08 *R. berlandieri*	1	LVT 4594	11	000	010101110	000	00101	111111	011011001	10010101	000	10111100	0111
09 *R. pipiens*	7	All Samples (except RDJ 903)	01	001	110111111	110	01111	011010	111010110	11011100	000	01101100	0010
10 *R. pipiens*	1	RDJ 903	01	001	110111111	110	01111	011010	111010110	11001100	000	01101100	0010
11 *R. blairi*	8	All Samples	11	000	010101110	000	00101	111110	011010010	10010101	000	11111100	0111
12 *R. chiricahuensis*	2	All Samples	11	000	missing	001	00100	missing	missing	10000100	001	01100101	0011