Resurrecting an extinct species: Archival DNA, taxonomy, and conservation of the Vegas Valley leopard frog.

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Abstract:

Suggestions that the extinct Vegas Valley leopard frog (*Rana fisheri = Lithobates fisheri*) may have been synonymous with one of several declining species have complicated recovery planning for imperiled leopard frogs in the southwestern United States. To address this concern, we reconstructed the phylogenetic position of *R. fisheri* from mitochondrial and nuclear sequence data obtained from century-old museum specimens. Analyses including representative North American *Rana* species placed archival specimens within the clade comprising Federally Threatened Chiricahua leopard frogs (*Rana chiricahuensis = Lithobates chiricahuensis*). Further analysis of Chiricahua leopard frogs recovered two diagnosable lineages. One lineage is composed of *R. fisheri* specimens and *R. chiricahuensis* near the Mogollon Rim, while the other encompasses *R. chiricahuensis* populations to the southeast. These findings ascribe *R. chiricahuensis* populations from the northwestern most portion of its range to a resurrected *R. fisheri*, demonstrating how phylogenetic placement of archival specimens can inform recovery and conservation plans, especially those that call for translocation, re-introduction, or population augmentation of imperiled species.

Keywords: Archival DNA, museum specimens, *Rana fisheri*, *Rana chiricahuensis*, taxonomy, conservation genetics.

Introduction

Conservation of imperiled species requires correct diagnosis of taxonomic status for effective implementation of management actions. The need for reliable taxonomy is most obvious in management plans that involve translocation, re-introduction, population augmentation, or

captive propagation (Kleiman 1989) and the declining leopard frog species (family Ranidae) from southwestern North America present an example of how uncertain taxonomic status can impede or complicate conservation strategies (e.g. Jaeger *et al.* 2001; Goldberg *et al*. 2004).

Addressing taxonomic concerns has, until recently, been considered 'too little, too late' for the extinct Vegas Valley leopard frog (*Rana fisheri* Stejneger 1893 = *Lithobates fisheri*) (Jennings & Hayes 1994). The species was known only from southern Nevada at four localities in the Las Vegas Valley, with individuals last collected in 1942 (Stebbins 1951). Introduced species (particularly bullfrogs, *Rana catesbiana* [= *Lithobates catesbeianus*]) and the loss of spring-fed habitats likely contributed to *R. fisheri*'s demise (Stebbins 1951). Plans to recover leopard frog populations within Las Vegas Valley have been complicated by suggestions that, based upon morphological similarities (Jennings 1988, Hillis & Wilcox 2005), *R. fisheri* may have been synonymous with either (1) the relict leopard frog (*R. onca* [= *Lithobates onca*]; Jaeger *et al.* 2001) known from sites in close proximity to the Las Vegas Valley; or (2) the Chiricahua leopard frog (*R. chiricahuensis* Platz & Mecham 1979 = *Lithobates chiricahuensis*), which has its closest populations 400 km distant along the Mogollon Rim of central Arizona (Platz & Mecham 1979).

Both *R. onca* and *R. chiricahuensis* have experienced dramatic population declines and range contractions (Bradford *et al.* 2004; Sredl & Jennings 2005). *Rana chiricahuensis* is now listed as Federally Threatened under the U.S. Endangered Species Act and *R. onca* is managed under a voluntary conservation agreement. Management plans for both species rely on expansion or re-establishment of populations. Accordingly, both species may be candidates for establishment in the Las Vegas Valley. The uncertain taxonomic status of extinct *R. fisheri*, however, raises questions about whether such an action would represent a translocation of an imperiled species to nearby vacant habitat or a re-introduction of a threatened species into former habitat. Herein, we present the first genetic analysis of *R. fisheri* from century-old archival

museum specimens to address alternative taxonomic hypotheses, and in so doing, to advance the recovery of imperiled leopard frogs across southwestern North America.

Material and Methods

Archival samples

Tissues were sampled from 33 historic *R. fisheri* specimens housed at the California Academy of Sciences (Supplementary Table 1). Of these samples, collections made in 1913 (Van Denburgh & Slevin 1921) were preserved in ethanol while those from 1938 were preserved in formalin. During tissuing, surgical utensils and work areas were wiped with DNAaway (Molecular Bioproducts) between samples.

DNA from *R. fisheri* tissue samples was extracted using a DNeasy tissue kit (Qiagen) according to the manufacturer's instructions with the following modifications: tissue samples were first soaked for 36 hours with three changes of PBS at 12 hour intervals. During tissue digestion, 5µl of dithiothreitol was added along with proteinase K to enhance protein digestion. DNA was initially selectively bound to the DNeasy membrane and then eluted from the membrane using manufacturer provided buffer heated to 56ºC and left to sit in the column for 20 minutes prior to centrifugation. All elutions were performed twice with 80 µl of buffer provided with the kit. Extractions from contemporary samples (collected 1980-2009) were carried out as per manufacturer recommendations in separate facilities.

All processing (extraction and amplification) of archival samples from 1910-1939 took place in a separate, clean facility with protocols recommended for use with degraded or ancient DNA (Gilbert *et al* 2005). All pre- and post PCR handling was also separated and positive and negative controls were used during PCR setup. Archival tissue samples were re-extracted, amplified and sequenced in triplicate for verification. Only those samples with triplicate confirmation of sequence data were used in the analyses.

Contemporary Samples

In order to explore the phylogenetic affinity of the archival *R. fisheri* specimens, we needed to provide a comparative sequence library for representative Southwestern and western ranid frog species. We used a combination of Genbank accessioned sequences (Dataset I, Hillis and Wilcox 2005, Goldberg *et al*. 2004) and sequences generated from ranid tissue samples collected during recent surveys (>1980) (Supplementary Table 2a).

Unpublished data for a large set of *R. chiricahuensis* samples collected as part of a separate project, was made available for our use (Data set III, n=229, Supplementary Table 2c). These samples were processed, including extraction and data generation, entirely at University of Arizona, Tucson. For the current project a subsample of DNA templates from that collection (Dataset II, n= 26, Supplementary Table 2b) were used to generate data for additional gene regions at Tulane University. These samples were processed after completion of the archival specimen data collection.

Markers and sequencing

Oligonucleotide primers were designed from published *R. onca*, *R. chiricahuensis* and *R. pipiens* sequences to amplify short (*ca.* 200bp) fragments of mitochondrial (mtDNA) 12s, Control Region (CR), and the nuclear Rhodopsin exon 1 region (Table1). Primers incorporated base ambiguities to increase possible amplification of the anonymous *Rana fisheri* DNA. All amplifications were performed on either a Perkins-Elmer or MJ Research thermocycler in 20–25 ml volumes. Cocktails included a PCR "Illustra puretaq READY-TO-GO" bead (GE Healthcare), 2-4 nmole template DNA; 2 μM mixed forward and reverse primer, with ddH_2O to volume. PCR parameters included initial denaturing of 4 min at 94ºC, followed by a 7 min extension with 31 subsequent cycles of 1 min at 94ºC, 1 min at 48–59ºC and 1.5 min at 72ºC,

followed by a final 4 min extension at 72ºC. Amplicons were purified using ExoSAP-It (USB). Forward and reverse cycle sequencing reactions were performed using BigDye chemistry, and analyzed on an ABI3100 automated sequencer (Applied Biosystems). Raw sequence files were edited, assembled, and aligned with Sequencher 4.9 (Gene Codes). Individual marker datasets were compiled and aligned individually in MEGA4 (Tamura et al. 2007) utilizing Clustal W (Larkin et al. 2007) (Gap penalties = 50, Gap Extension penalties = 25) and checked by eye prior to concatenation.

Analyses

We used an iterative approach to assess the taxonomic affinity of *R. fisheri*. Using MRBAYES, v.3.0b3 (Huelsenbeck & Ronquist 2001), we performed phylogenetic analyses of two datasets comprised of: (I) 12s for *Rana fisheri* and North American Ranidae Genbank sequences (Hillis and Wilcox 2005, Supplementary Table 2a); and (II) combined short, mtDNA (Control region and 12s) and nuclear (Rhodopsin exon 1) regions for *Rana fisheri, R. Chiricahuensis* from a broad geographic distribution, and Genbank sequences other representative southwestern ranid species (Hillis & Wilcox 2005, Frost *et al* 2006; Supplementary Table 2b). Each marker for the combined dataset (II) was initially analyzed independently with the substitution model specified by MR. MODELTEST v.2.3 (Posada & Crandall 1998) and then according to the HKY85+I+G and GTR+I=G models, respectively. The Markov Chain Monte Carlo searches were run with 5 chains for 10,000,000 generations with trees sampled every 500 generations (the first 20,000 trees were discarded as "burnin") and assessed using TRACER v1.4.1 (Rambaut & Drummond 2005). We used the program CAOS (Characteristic Attribute Organization System; Sarker and DeSalle 2008) to explore patterns of character distribution across the resulting phylogenetic hypothesis.

Finally, we used Network 4.5 (Fluxus) to construct a Median Joining network for an additional, expanded dataset (dataset III) of CR sequences (n = 229, Supplementary Table 2c) from an ongoing study of *R. chiricahuensis*. The structure of the resulting network was evaluated according to phylogenetic relationships recovered from analyses of the combined dataset.

Results

We successfully extracted DNA from 15 ethanol preserved, archival specimens of *R. fisheri* (Supplementary Table 1). We were unable to recover usable DNA from formalin preserved samples. The amplification success of individual specimens varied across gene regions. However, we were able to generate a minimum of 3 consistent sequences for each target gene region from between 5 and 15 individual specimens of *R. fisheri* (Table 1). As these sequences were invariant, we included 2 representative sequences in all subsequent analyses.

The broad comparison of 12s mtDNA sequences from the entire collection of representative North American ranid frogs (Dataset I) placed *R. fisheri* within *R. chiricahuensis*, and as a distant relative of *R. onca* (Figure 1a). Strong support was found for a sister relationship between the *R. fisheri–R. chiricahuensis* clade and "*Rana* Species2" from San Louis Potosi, Mexico (Hillis & Wilcox 2005). Our results agree with those from prior studies (Jeager et al. 2001, Olah-Hemmings et al. 2010), wherein *R. onca* was recovered as the sister species of *R. yavapaiensis* (= *Lithobates yavapaiensis*), the lowland leopard frog. Additional support for this relationship came from alignment of the nuclear Rhodopsin exon 1 gene region, which indicated a 4bp difference between *R. fisheri* and *R. onca* samples and no differences between *R. fisheri* and *R. chiricahuensis*.

Bayesian and ML phylogenetic analyses of the combined dataset (II) using *R. onca* and *Rana pipiens* as outgroups revealed two clades: one consisting of *R. fisheri* plus *R. chiricahuensis* from the northwestern portion of it's range near the Mogollon Rim, and another derived *R. chiricahuensis* clade including populations to the south and east (Figure 1b). The

CAOS analysis revealed the presence of 8 pure diagnostic and 6 private characters for the *R. fisheri*+ northwestern chiricahuensis clade; and 7 pure diagnostic and 9 private characters for the southestern+subaquavocalis clade (Table 2). Thus, mtDNA and nuclear sequences for the combined *R. chiricahuensis* dataset (II) revealed the presence of fixed, diagnostic characters indicative of disrupted gene flow between two population aggregates (Davis and Nixon 1992).

Haplotype network analysis of the larger Control Region dataset (III) recovered 17 haplotypes in two evolutionary lineages that correspond to the clades recovered in the analysis of the combined dataset (Figure 2). The lineage that includes *R. fisheri*, which is differentiated from all other haplotypes by 7 substitutions, is distributed across the Mogollon Rim. Of the 55 localities included, only two harbor haplotypes from both CR lineages (Figure 2).

Discussion

Genetic analysis of archival museum specimens has proven useful for determining the validity of taxonomic distinctions for imperiled and declining taxa (Bouzat *et al.* 1998, Goldstein & DeSalle 2003). In this study, we examined archival specimens to resolve the taxonomy of *R. fisheri*—an extinct species—to advance recovery planning for leopard frog populations in southwestern North America. Phylogenetic analyses of nuclear and mtDNA sequence variation among century-old specimens placed *R. fisheri* within extant populations of *R. chiricahuensis.* Analyses of mtDNA variation indicate that specimens of *R. fisheri* in combination with *R. chiricahuensis* individuals from the northwestern potion of that species' range represent a diagnosably distinct lineage within *R. chiricahuensis*; a finding which is consistent with prior genetic analyses that distinguish between Mogollon Rim populations of *R. chiricahuensis* and populations in southern Arizona (Goldberg *et al*. 2004). According to nomenclatural priority, we therefore suggest that, pending further analyses, the northwestern lineage of *R. chiricahuensis* is referable to the previously described, extinct, species, *R. fisheri* Stejneger 1893.

The phylogenetic placement of *R. fisheri* from the Las Vegas Valley within northwestern Mogollon Rim populations of *R. chiricahuensis* (400 km distant) parallels biogeographic distributions of other species in the region (Lomolino *et al.* 1989). Within leopard frogs, a divergent lineage of *R. yavapaiensis* occurs along the Colorado River in the western Grand Canyon (east of Las Vegas Valley), disjunct from other populations along the Mogollon Rim (Olah-Hemmings *et al.* 2010). The distribution and connectivity of habitats for vertebrate species in this region appear to have been greatly impacted by climatic change, possibly at scales less pronounced than those associated with glacial-interglacial cycles (Jaeger *et al.* 2005). Further phylogeographic studies and ecological niche modeling (e.g., Raxworthy *et al.* 2007), may provide valuable insight in resolving this pattern, and also help identify important areas of connectivity in the changing arid southwest.

The unexpected resurrection of *R. fisheri* via phylogenetic placement of archival specimens highlights the utility of museum collections to provide evidence of pre-anthropogenic-disturbance conditions and better defines paths toward recovery of imperiled leopard frogs in southwestern North America. Although the Chiricahua leopard frog may remain a valid taxon in some portion of its current range (Goldberg *et al.* 2005), our data indicate that, at minimum, northwestern populations of the species are now referable to *R. fisheri*. Accordingly, recovery plans, especially those that involve re-introductions into former habitat, should be reviewed in light of these findings.

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Figure and Table captions:

Table 1. Primers and sample sizes of historic *Rana fisheri* specimens (Supplementary Table1) and congeners by gene region.

Table 2. CAOS results indicating sites within gene regions for diagnostic pure and private nucleotide characters.

Figure 1. 50% majority rule consensus trees depicting results of Bayesian phylogenetic analysis of archival *Rana fisheri* based on (a) 12s mtDNA for published North American ranid frogs (Hillis and Wilcox 2005; Dataset (I); Supplementary Table 2a) and (b) combined 12s, Control Region and Rhodopsin exon 1 within *R. chiricahuensis* (Dataset (II); Supplementary Table 2b).

Figure 2. Distribution of Control Region *R. fisheri* (red) and *R. chiricahuensis* (black) haplotype groups across 55 sample sites in the southwestern United States and Mexico, as presented in Supplementary Table 2c . Shading = elevation contours from lower (white) to higher elevations (dark grey); black lines = state boundaries; blue lines = major rivers of geographical reference. (Inset) Control Region haplotype network for dataset (III) with individual haplotypes as presented in Supplementary Table 2c, scaled according to frequency.

Figure 1a.

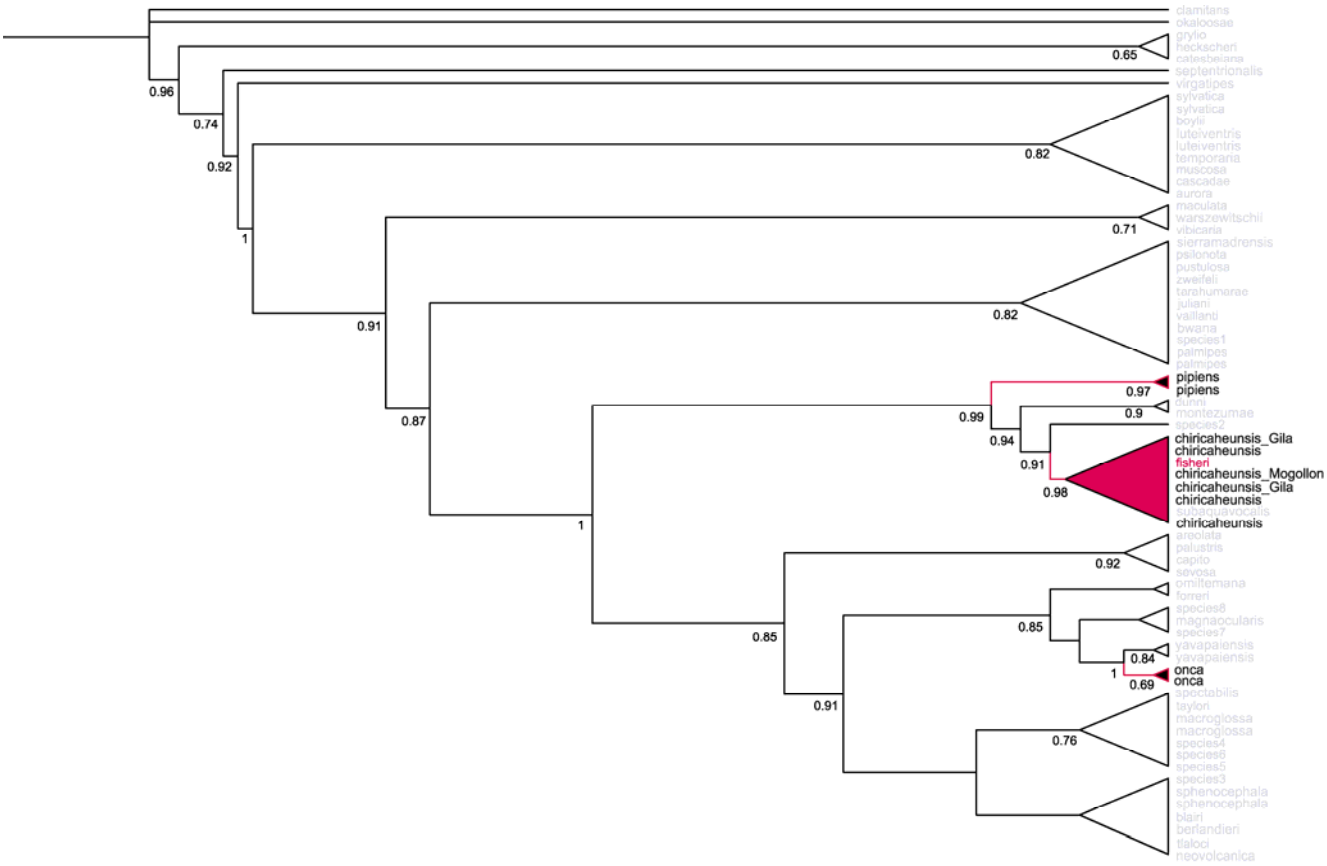
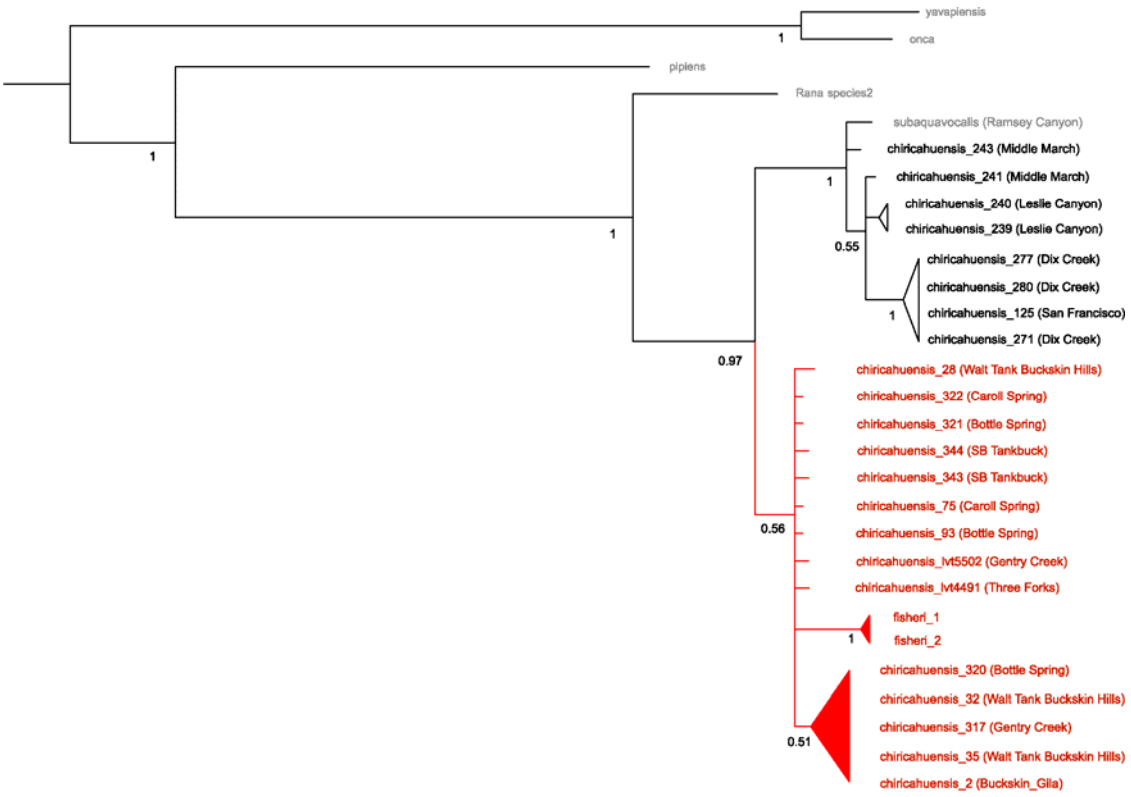
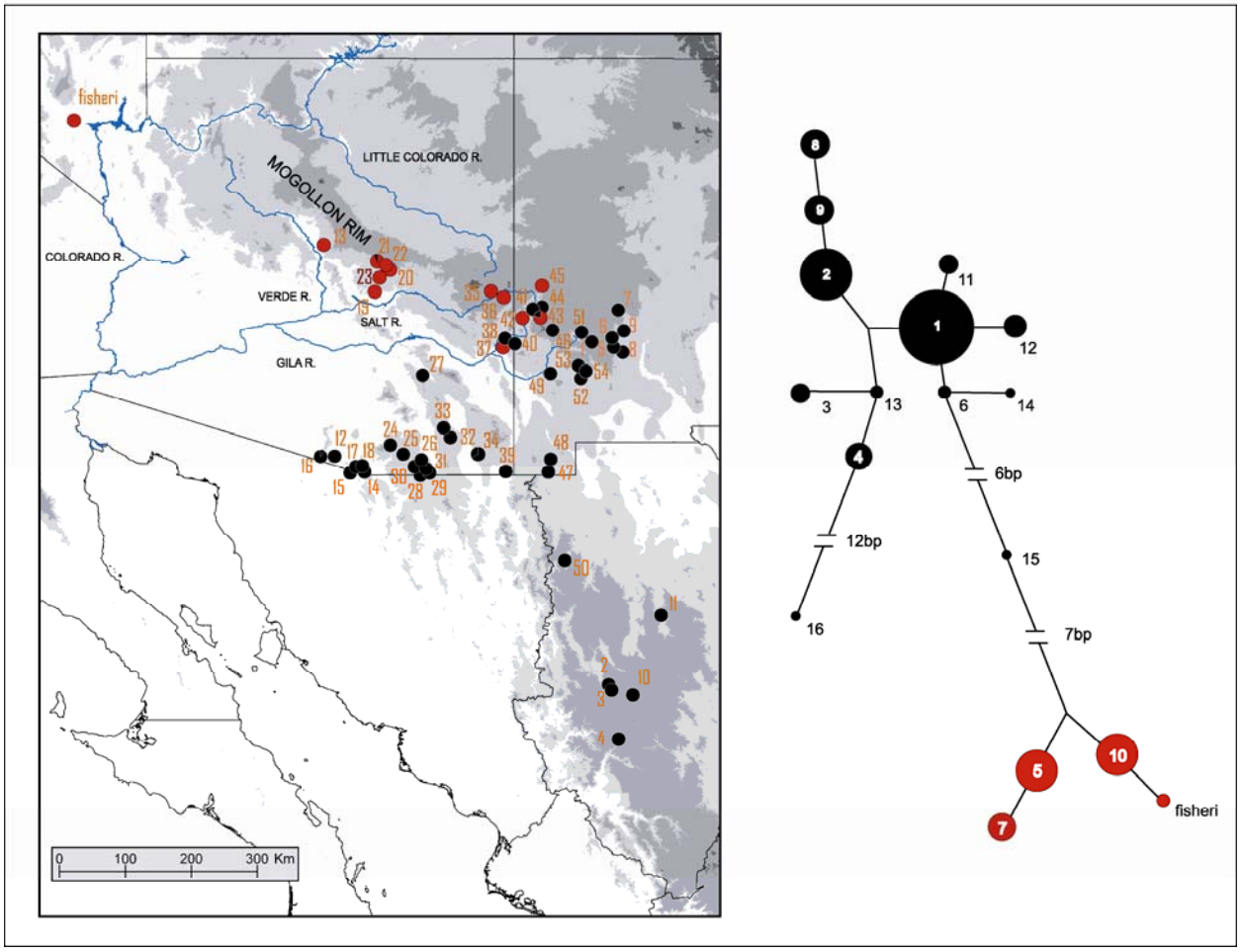


Figure 1b.




fisheri
LITTLE COLORADO R.
MOGOLLON RIM
COLORADO R.
VERDE R.
SALT R.
GILA R.
0 100 200 300 Km
8
9
2
11
1
12
3
13
6
14
4
6bp
12bp
15
16
7bp
10
5
7
fisheri

1

Table 1.

Gene Region	Primer 5'-3' Sequence	n=				Total Basepairs
		Rf	*Rc*	*Ro*	*Rp*	1191
Control Region		11	26	1	1	281
RfcrF1	5'-ATTAAGTACCCCATATTATGCTTTCT-3					
RfcrF2	5'-TGGTTTAATTTATATACATATT-3'					
RfcrF3	5'-TGTATTAATCTATTTATGTCT-3'					
RfcrR1	5'-TATACATGTAAGTACTAATGC-3'					
Rhodopsin Exon		7	20	1	na	278
RhodF	5-TCAGTATTACCTGGCAGAGCCATGG-3					
Rhod1A	5'-ACCATGAACGGAACAGAAGGYCC-3					
Rhod1C	5'-CCAAGGGTAGCGAAGAARCCTTC-3					
Rhod1D	5'-GTAGCGGAAGAARCCTTCAAMGTA-3					
12s		15	23	1	2	632
Ro12s216F	5'-CAAYACGTCAGGTCAAGGTG-3					
Ro12s460R	5'-CYTGTTTCGACTTGCCTCTT-3					

Rf=Rana fisheri, Rc=Rana chiricahuensis, Ro=Rana onca, Rp=Rana pipiens

Table 2. Results of CAOS results indicating sites within gene regions for diagnostic pure and private nucleotide characters

Pure Diagnostic		Alignment Position										Total
		12s	CR									
grp	1 Fisheri+NW	463	695	730	780	781	836	838	843			8
grp	2 Rsubaq+SE	316	836	838	844	848	856	905				7
Private Diagnostic												
		12S			CR							
grp	1 Fisheri+NW	92	346	530	735	740	762					6
grp	2 Rsubaq+SE	191	383	633	698	711	713	732	771	789	789	10