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TAXONOMIC STATUS AND PHYLOGENY OF THE SAN BERNARDINO SPRING SNAIL POPULATIONS INTO THE GENUS *PYRGULOPSIS* IN SONORA AND ARIZONA

ESTATUS TAXONÓMICO Y FILOGENIA DE LAS POBLACIONES DEL CARACOL DE MANANTIAL DE SAN BERNARDINO AL INTERIOR DEL GÉNERO *PYRGULOPSIS* EN SONORA Y ARIZONA

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ABSTRACT

The San Bernardino springsnail (*Pyrgulopsis bernardina*) inhabits springs in the upper San Bernardino River (SBR) basin in southeastern Arizona and northeastern Sonora. Loss of populations in Arizona associated with habitat degradation has resulted in concern for the continued persistence and viability of the species. Springsnails at the Arroyo San Bernardino (ASB) sub-basin in Sonora are considered *P. bernardina* but this has not been validated through genetic studies to receive legal protection. The species of *Pyrgulopsis* detected in the Arroyo Cajon Bonito (ACB) sub-basin in Sonora sites is not known. The goal of this study is to provide genetic information to resource managers to clarify the taxonomic relationships between populations of *Pyrgulopsis* in the upper SBR basin in Sonora with those historically found at the type locality in Arizona based on cytochrome oxidase 1 (COI) gene fragment. COI fragments from the two sub-basins samples in Sonora, confirms that *Pyrgulopsis* are conspecific with type locality samples from Arizona. Maximum parsimony and maximum likelihood analysis of all populations, agree with the current systematics of the species. Monophyly of all populations of *P. bernardina* was observed, and populations within each sub-basin form two monophyletic clades. Conservation planning should recognize the two sub-basins genetic divergence. Within the ASB clade, genetic relationships suggest historical connectivity with the type locality in Arizona.

Keywords: Mitochondrial COI sequences, Gastropoda, Hydrobiidae.

RESUMEN

El caracol de manantial de San Bernardino (*Pyrgulopsis bernardina*) habita manantiales en la cuenca alta del Río San Bernardino (RSB). La pérdida de poblaciones en Arizona está asociada con la degradación de hábitat y existe preocupación por la persistencia y viabilidad de la especie. Los caracoles de manantial de la sub-cuenca del ASB son considerados *P. bernardina*, pero no ha sido validado genéticamente para recibir protección legal. Se desconoce la especie de *Pyrgulopsis* de la sub-cuenca del ACB. El objetivo de este trabajo es proveer información genética a manejadores de recursos para clarificar las relaciones taxonómicas entre las poblaciones de *Pyrgulopsis* en el RSB con los históricamente encontrados en la localidad tipo en Arizona basados en un fragmento del gen COI. Los fragmentos del COI de las dos sub-cuencas confirman que los *Pyrgulopsis* son conespecíficos con las muestras de la localidad tipo en Arizona. Los análisis de máxima parsimonia y máxima verosimilitud de todas las poblaciones concuerdan con la sistemática de la especie. Se observó monofilia para todas las poblaciones, y las poblaciones dentro de cada sub-cuenca forman dos clados monofiléticos. Los planes de conservación deberán reconocer esta diferencia genética entre ambas sub-cuencas. Dentro del clado del ASB, las relaciones genéticas sugieren conectividad histórica con la localidad tipo de Arizona.

Palabras clave: Secuencias COI Mitocondrial, Gastropoda, Hydrobiidae.

INTRODUCTION

The San Bernardino springsnail (*Pyrgulopsis bernardina* Taylor, 1987) inhabits, or is presumed to inhabit, spring and spring-associated habitats in the upper San Bernardino River (SBR) basin of southeast Arizona and northeast Sonora. The species was initially described from specimens collected by Dwight Taylor in 1968 at a spring in Cochise County, Arizona, later named Snail Spring by Malcom et al. (2003) and referred to as San Bernardino Spring by Hurt (2004). Between about 2003 and 2005, drying conditions exacerbated by the local extraction of ground water, led to the extirpation of springsnails at the type locality Snail Spring (SS) on the San Bernardino National Wildlife Refuge (SBNWR, Figure 1) (Varela-Romero and Myers, 2010).

In Arizona, San Bernardino springsnails, or what is assumed *P. bernardina*, also have been reported from springs in the immediate vicinity of the type locality Snail Spring on the John Slaughter Ranch, adjacent to San Bernardino National Wildlife Refuge (SBNWR). Presently, in Arizona, the presumed species is only known to occur at Goat Tank Spring (GT).

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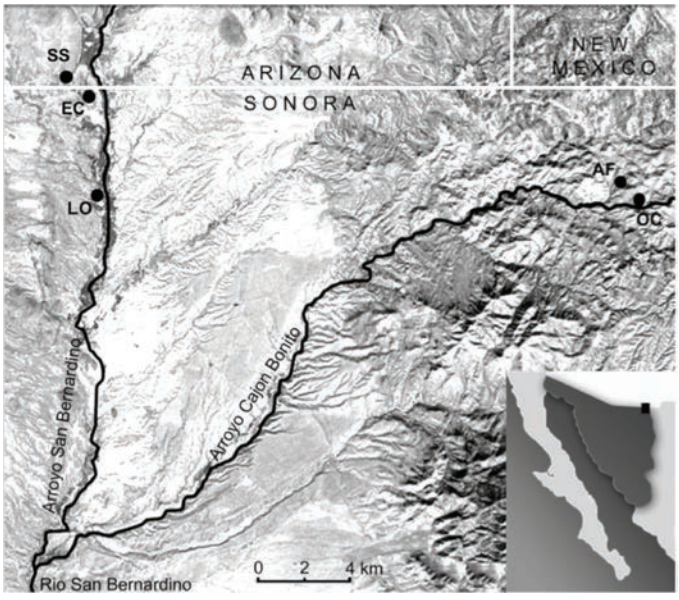

Figure 1. General location of study area in southeastern Arizona and northeastern Sonora. Black spots indicate sampling sites of the Sonoran populations of *Pyrgulopsis bernardina* in the San Bernardino River drainage. Snail Spring = SS, El Chorro = EC, Los Ojitos = LO, Agua Fria = AF, Ojo Caliente = OC, San Bernardino National Wildlife Refuge = SBNWR

Figura 1. Localización general de área de estudio en el sureste de Arizona y noreste de Sonora. Los puntos negros indican los sitios de muestreo de las poblaciones sonorenses de *Pyrgulopsis bernardina* en la cuenca del Río San Bernardino. Snail Spring = SS, El Chorro = EC, Los Ojitos = LO, Agua Fria = AF, Ojo Caliente = OC, San Bernardino National Wildlife Refuge = SBNWR

In Sonora, downstream from the type locality, springsnails have been collected or reported from spring sites in the Arroyo San Bernardino basin springs at El Chorro (EC), Los Ojitos (LO), and El Ojito (EO). Springsnails from EC, and LO were determined by Robert Hershler as *P. bernardina* presumably on the basis of diagnostic anatomical and morphological characteristics presented by Taylor (1987), Hershler and Landye (1988) and Hershler (1994). Elsewhere in the upper San Bernardino River basin, springsnails have been found in two sub-basins of the Arroyo Cajon Bonito drainage: Arroyo Agua Fria and Arroyo Ojo Caliente. Collections from one spring in Arroyo Agua Fria (AF) have been identified on the basis of the morphology by Robert Hershler as "*Pyrgulopsis* sp." Additional observations of springsnails in Arroyo Ojo Caliente (OC) have not been formally collected or identified (Figure 1).

Hurt (2004) evaluated a segment of the cytochrome oxidase 1 (CO1) gene of springsnails collected from the type locality (SS), just prior to their extirpation, as part of a regional assessment of genetic patterns among *Pyrgulopsis* populations from the lower Colorado River. Liu and Hershler (2005), in their genetic study including mitochondrial DNA sequences from COI, NADH dehydrogenase I (ND1), and 16S rRNA (16S) genes of *Pyrgulopsis* populations from western North America, included specimens obtained during 2002 from El Chorro Spring in the San Bernardino River watershed of northern Sonora (USNM 1010780), and designed as *P.* cf. *bernardina*.

The taxonomic status of springsnails in Sonora previously identified on the basis of morphology as *P. bernardina* has not been verified through genetic comparison with those from the type locality in Arizona. Although specimens from EC and LO have been identified as *P. bernardina* based on anatomical and morphological characteristics, genetic validation of this determination is not conclusive. The availability of COI sequences from springsnails formerly inhabiting the type locality (Hurt, 2004) provides an opportunity to evaluate the relationship between these various populations in Sonora with a known population of *P. bernardina*.

Based in large part on the loss of populations in Arizona associated with habitat degradation, and reflecting concern for the continued existence of the species, the United States Fish and Wildlife Service (USFWS) recently protected *P. bernardina* as a threatened species under the Endangered Species Act (USFWS, 2008, 2009, 2010).

With the extirpation of San Bernardino springsnails from the type locality in Arizona, and the range of the species in Arizona limited to one site (GT), opportunities for the restoration of the species in Arizona would be greatly enhanced if springsnails inhabiting in Sonora were found to be genetically indistinguishable from those from the type locality. Resource managers in both Mexico and the United States must know with more certainty whether or not the species still exists in Sonora and how closely these populations are related, especially to the population that used to inhabit the type locality. The goal of this paper is to provide genetic information to resource managers to clarify the taxonomic relationships between populations of *Pyrgulopsis* in the upper San Bernardino River basin.

MATERIALS AND METHODS

Sampling

We visited sites historically occupied by *Pyrgulopsis* on May 2010. At EC, LO, AF, and OC sites, we searched for *Pyrgulopsis* in different microhabitats. Various substrates were sampled with small-mesh (<1mm) hand nets (benthos), or visually inspected (rocks, pieces of organic matter, plants) for the presence of springsnails and other aquatic organisms. At each site where we encountered *Pyrgulopsis*, we attempted to collect 30 individuals of various sizes for genetic analysis, and transferred to 100% ethanol (Table 1). All specimens were deposited in the Molecular Ecology laboratory of the Department of Scientific and Technological Research of the University of Sonora (DICTUS by its Spanish acronym). Permit for collections were issued by Secretaría del Medio Ambiente y Recursos Naturales SGPA/DGVS/00505/10 to the first author.

Table 1. Collection localities of samples analyzed in this study
Tabla 1. Localidades de recolecta de muestras analizadas en el presente estudio

Species	Localities	Catalog number	Lat/long (degress and minutes)
Pyrgulopsis sp.	Yaqui Basin, Bavispe river sub-basin, Arroyo Cajon Bonito, Arroyo Agua Fria (AF), seep on the east side of the arroyo, downhill of the road to Los Ojos Ranch. (AV-290410-3).	USON-0100	31° 17,5' N 108° 59,7' W
Pyrgulopsis sp.	Yaqui Basin, Bavispe river sub-basin, Arroyo Cajon Bonito, El Ojo Caliente (OC) (northwest from the cabin) on Los Ojos Ranch. (AV-290410-5).	USON-0101	31° 17,0' N 108° 59,4' W
Pyrgulopsis bernardina	Yaqui Basin, Bavispe river sub-basin, Arroyo San Bernardino, Los Ojitos (LO), south of Mexico Highway 2, on the west side of the Arroyo San Bernardino. (AV-300410-6).	USON-0102	31° 18,0' N 109° 15,7' W
Pyrgulopsis bernardina	Yaqui Basin, Bavispe river sub-basin, Arroyo San Bernardino, downstream of Manantial El Chorro (EC). (AV-300410-6).	USON-0103	31° 19,8' N 109° 16,0' W

USON = University of Sonora, DICTUS www.dictus.uson.mx
USON = Universidad de Sonora, DICTUS www.dictus.uson.mx

DNA Extraction, Amplification, and Sequencing

Total DNA was extracted from total snails body, previous discard of the mayor portion of the shell, following the protocol of the QIAamp DNA Mini Kit. Amplifications of the COI (700 bp) were done using the universal PCR primers (Folmer et al., 1994), (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). Amplifications were conducted using puReTaq Ready-to-Go PCR beads (GE Healthcare) in a 25 µl total volume, containing 2,5 puReTaq DNA polymerase, 200 µM of each dNTP in 10 mM Tris-HCl (pH 9,0), 50 mM KCl, 1,5 mM MgCl2, and 1,25 µ1 of each primer (10 M), approximately 30 ng of total DNA 1 µl of template (2 µl), and 20,5 µl of sterile water. Thermal cycling was performed using the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 40°C for 1 min, 72°C for 2 min, and finally one cycle of 7 min at 72°C. All individual PCR products were completely sequenced in both strands by commercially company (Macrogen Inc, Korea).

Total DNA of three museum samples from AF was extracted at the Conservation Genetics Laboratory of the University of Arizona, following spin-column protocol for animal tissues detailed in the DNeasy Blood & Tissue Handbook of QIAGEN. The protocol for one sample was modified slightly by soaking the snail in EDTA overnight, followed by two rinses in water, two rinses in ethanol, and allowing the snail to dry prior to beginning the extraction process.

Sequence Analyses

Nucleotide composition of COI fragment was determined using GeneQuest (DNAstar, Madison, WI). BLASTN searches against GenBank data for all sequences resulted in high homology to the corresponding genes from *Pyrgulopsis* species. Nucleotide sequences were aligned and edited using EditSeq and MegAlign (DNAstar, Madison, WI). We used sequences from GenBank (AY485595, AY485596, AY485597) developed by Hurt (2004) from *Pyrgulopsis bernardina* collected from the type locality (SS) as a baseline genome describing the species. In addition, we used sequences from GenBank from the analysis of *Pyrgulopsis cf. bernardina* (AY627951) by Liu and Hershler (2005) collected in 2002 from El Chorro, and a specimen from the Smithsonian National Museum of Natural History (NMNH 1074396). Sequences from our study were deposited in the GenBank (*P. bernardina* EC haplotype 1 KC417419, haplotype 2 KC417420, and haplotype 3 KC417421; LO haplotype 1 KC417422, haplotype 2 KC417423, and haplotype 3 KC417424; AF haplotype 1 KC417426, haplotype 2 KC417427, and haplotype 3 KC417428; NMNH KC417425; OC haplotype 1 KC417429, haplotype 2 KC417430, and haplotype 3 KC417431). Sequences from other springsnail species were used as external group obtained from GenBank (*P. trivialis* AY485559, *P. hubbsi* AY627918, *P. gilae* AY627952, *P. archimedix* AY426355, *P. arizonae* AY627948, and *Cincinnatia integra* (AY627916).

Phylogenetic Analysis

The construction of phylogenetic hypotheses from the dataset was done using maximum parsimony (MP), and maximum likelihood (ML). For MP, we obtained the most parsimonious tree or trees with tree bisection-reconnection (TBR) branch-swapping heuristic searches in PAUP* version 4.0b10 (Swoford, 2002) in which, all characters were equally weighted and starting trees were obtained by 1000 random

stepwise additions. Nodal support was estimated by calculation of non-parametric bootstrap (1000 pseudo-replicate, 10 random addition) proportions (Felsenstein, 1985) and decay indices (Bremer, 1994) using PAUP* and the software application TreeRot. The hierarchical likelihood ratio tests (hLRTs) and additionally calculated Akaike Information Criterion (AIC) values in MrModelTest2.2 (Nylander, 2004), a modified version of ModelTest (Posada and Crandall, 1998), were conducted to determine the optimal model of nucleotide substitution in our ML analysis. We calculated tree topology and parameter values in a ML search using the software application PHYML v.2.4.4 (Guindon and Gascuel, 2003). A branch-swapping search for a tree of higher likelihood was performed with the input recovered parameters and tree into PAUP*. This tree was used as the starting tree for a non-parametric bootstrap analysis of the data in PHYML (1000 pseudoreplicates, settings as for initial tree search) to estimate support for the nodes of the ML tree. Trees constructed were generated using the software FigTree v1.2.3 (http://tree.bio.ed.ac.uk/).

RESULTS AND DISCUSSION

We obtained partial sequences of the COI gene from twelve specimens obtained of each sample sites (Ojo El Chorro, Los Ojitos, Ojo Agua Fria, and Ojo Caliente), and one sequence collected from AF and deposited at the NMNH. After alignment, 578 bp of COI from *Pyrgulopsis* were obtained. The MP and ML criteria (methods) were used to test the variability of the COI fragments in the Sonoran populations of *Pyrgulopsis* (MP: tree length = 578 steps, c. i. = 0,639, r. i. = 0,788; and MP: tree length = 2102 steps). Ninety three of the 578 sites aligned were parsimony informative.

Maximum parsimony and maximum likelihood criteria show that Sonoran specimens form a monophyletic clade with all the *P. bernardina* and *P*. cf *bernardina* sequences reported in GenBank and from specimen collected by Liu and Hershler (2005) (from sites in Arizona and Sonora, Figures 2 and 3). A parsimony consensus tree from 4 trees and ML tree shows a Sonoran specimens clade (our samples) sharing a common ancestor with the *P. bernardina* GenBank sequences from both Arizona and Sonora sites (AY485595, AY485596, AY485597 from SS, and AY627951 from EC). Bootstrap supports in MP and ML trees were high (100 to 81 %) for all sample sites we sampled (Figure 2 and 3). Both analysis show two monophyletic groups, one from Arroyo San Bernardino (EC, LO) and other one in the Arroyo Cajon Bonito (AF, OC) with high statistical values, that support a genetic structure and geographic isolation of the Sonoran populations. The Sonoran and Arizona populations it shows into the analysis as two geographic clades representing two sub-basins into the Rio San Bernardino. Therefore, we suggest that populations of both sub-basins should be considered different Evolutionarily Significant Units (ESU) in the future conservation and management plans.

In addition, the specimens from AF and OC in the Arroyo Cajon Bonito were linked by the COI sequence of one

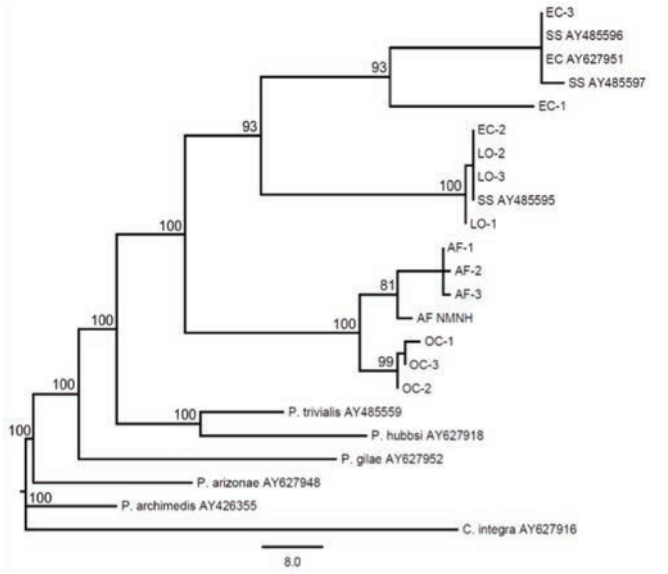

Figure 2. Maximum parsimony phylogenetic tree of specimens of *P. bernardina* and other closely related taxa using a fragment of the COI gene (578 bp). SS (Snail Spring; GenBank specimens SS AY485595, SS AY485596 and SS AY485597); EC (El Chorro; GenBank specimen AY627951); LO (Los Ojitos); AF (Agua Fria); OC (Ojo Caliente). Numbers above nodes indicate statistical support

Figura 2. Árbol filogenético de máxima parsimonia de especímenes de *P. bernardina* y otras especies cercanas, usando un fragmento del gen COI (578 bp). SS (Snail Spring; especímenes del GenBank SS AY485595, SS AY485596 and SS AY485597); EC (El Chorro; especimen del GenBank AY627951); LO (Los Ojitos); AF (Agua Fría); OC (Ojo Caliente). Los números sobre los nodos representan el boostrap

specimen from several collected in 2004 and deposited at the Smithsonian National Museum of Natural History (NMNH 1074396). This specimen at least represents a distinct haplotype not recorded in our samples. Analysis of the rest of the specimens could define the topology of the tree in both criteria. Besides, this analysis is supported by a partial sequence of the COI, limiting the inference by the use of the complete sequence of the gene. In the same way, the use of different molecular markers should support better topology of the trees that agree with the morphological inference. COI is the DNA barcoding gene most used in invertebrates and the specimen from El Chorro (EC AY627951) is the unique sequence deposited at the Global Online Database (BOLD: http://www.barcodinglife.org). The DNA barcoding libraries can support conventional taxonomic workflow by high-throughput identification of unknown specimens and by helping to draw attention to a new and cryptic species (Hajibabaei, et al., 2007). Future work can be directed to use the COI as a barcoding to build libraries of specimens like the Agua Fria and compare with the rest of the populations in the basin.

General topology of the trees, both criteria, shows the native springsnails collected in this study as monophyletic forming two subclades evidencing a hierarchical genetic

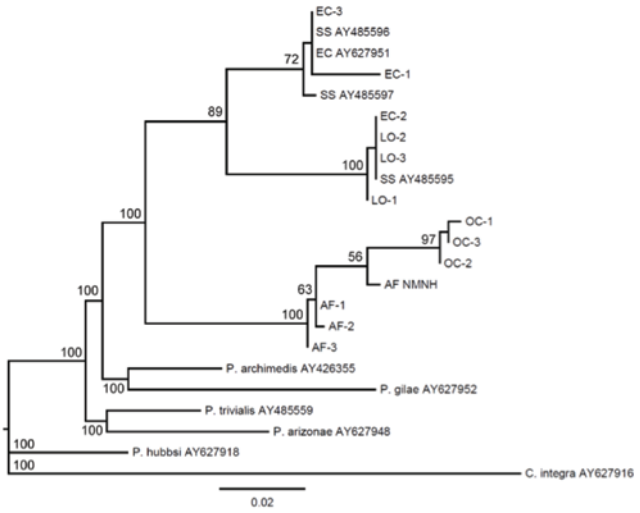

Figure 3. Maximum likelihood phylogenetic tree of specimens of *P. bernardina* and other closely related taxa using a fragment of the COI gene (578 bp). SS (Snail Spring; GenBank specimens SS AY485595, SS AY485596 and SS AY485597); EC (El Chorro GenBank specimen AY627951); LO (Los Ojitos); AF (Agua Fria); OC (Ojo Caliente). Numbers above nodes indicate statistical support

Figura 3. Árbol filogenético de máxima verosimilitud de especímenes de *P. bernardina* y otras especies cercanas, usando un fragmento del gen COI (578 bp). SS (Snail Spring; especímenes del GenBank SS AY485595, SS AY485596 and SS AY485597); EC (El Chorro especimen del GenBank AY627951); LO (Los Ojitos); AF (Agua Fría); OC (Ojo Caliente). Los números sobre los nodos representan el soporte estadísitico

structure in the Rio San Bernardino, supporting a natural clade in the genus *Pyrgulopsis*, and it is highly consistent with the relationships at genus level on the established systematic of Hurt (2004), and Liu and Hershler (2005). The previous hypothesis of evolutionary history of the genus was presented by Hershler (1994) and Hershler and Landye (1988) using morphological characteristics (reproductive characters), and Hurt (2004) and Liu and Hershler (2005) by mitochondrial genes (COI, ND1, 16S). Inconsistence in both types of data was observed in the entire genus, and shows *Pyrgulopsis* as a paraphyletic genus, mostly when the use of different markers where included. Hershler's morphological clades of the *Pyrgulopsis* were supported by the MP and ML inference in our study.

CONCLUSIONS

The COI fragment of our study results coherent with gene of *P. bernardina* deposited in the GenBank (SS), and reveal identity and monophyly of Mexican populations. Both MP and ML phylogenetic analysis of the Mexican populations, agree with the current systematics of the species.

Monophyly of the Sonoran populations in *P. bernardina* was observed for both Arroyo San Bernardino sub-basin (EC, LO), and Arroyo Cajon Bonito sub-basin (AF, OC).

P. bernardina analyzed that had been collected at the type locality in Arizona (Snail Spring) and in the Arroyo San Bernardino sub-basin in Sonora, it appears that should be considered conspecific with those from the type locality.

Based on our preliminary study, all populations of *Pyrgulopsis* in the upper Rio San Bernardino basin of Sonora, including Arroyo San Bernardino and Arroyo Cajon Bonito, should be considered *P. bernardina*. However, *P. bernardina* in these two sub-basins exhibit genetic differences that should be considered as distinct ESU during any conservation effort.

RECOMMENDATIONS

This preliminary study indicates that *P. bernardina* from the Arroyo San Bernardino sites in Sonora (EC, LO) are much more closely related to *P. bernardina* from the type locality in Arizona (SS) than the *P. bernardina* inhabiting the Arroyo Cajon Bonito sites of Sonora (AF, OC). Therefore, if translocations of the species are required to re-establish the species in Arizona, the source population should not come from sites in Arroyo Cajon Bonito, Sonora.

Develop studies to increase the knowledge of the morphology of the San Bernardino springsnail, and develop additional molecular markers to support phylogeny, management, and conservation.

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