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Short Communication

# Phylogenetic relationships of leopard frogs (*Rana pipiens* complex) from an isolated coastal mountain range in southern Sonora, Mexico

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ABSTRACT

Mitochondrial DNA sequence data from the control region and 12S rRNA in leopard frogs from the Sierra El Aguaje of southern Sonora, Mexico, together with GenBank sequences, were used to infer taxonomic identity and provide phylogenetic hypotheses for relationships with other members of the *Rana pipiens* complex. We show that frogs from the Sierra El Aguaje belong to the *Rana berlandieri* subgroup, or *Sarrilirana* clade, of the *R. pipiens* group, and are most closely related to *Rana magnaocularis* from Nayarit, Mexico. We also provide further evidence that *Rana magnaocularis* and *R. yavapaiensis* are close relatives.


## 1. Introduction

Molecular studies on North American leopard frogs [*Rana pipiens* complex, also referred to as the *Pantherana* clade (Hillis and Wilcox, 2005)] have helped clarify the taxonomy, evolutionary history and phylogenetic relationships among species belonging to this complex, and, in addition, have suggested the presence of additional, undescribed cryptic species, especially in Mexico and Central America (Jaeger et al., 2001; Goldberg et al., 2004; Hoffman and Blouin, 2004a; Zaldívar-Riverón et al., 2004; Hillis and Wilcox, 2005). The *R. pipiens* complex, previously thought to be comprised of a single wide-ranging species, probably includes >30 separate species (Hillis and Wilcox, 2005). Because morphological differences among species of leopard frogs are often subtle (Frost, 1982; Hillis, 1988), with individual characters sometimes showing considerable variability (Frost and Bagnara, 1976), confidence in species determinations can be enhanced by using molecular characters. Studies on the biodiversity, conservation and population monitoring of leopard frogs in Mexico and elsewhere clearly depend on accurate species determinations.

The Sierra El Aguaje in southern Sonora, Mexico (Fig. 1) is a small coastal range of desert mountains isolated from the Sierra Madre Occidental in eastern Sonora, with elevations reaching ~880 m above sea level. Although located in the Sonoran Desert, the flora of the protected canyons in the Sierra El Aguaje typically show tropical elements (Felger, 1999). The region is characterized by high summer temperatures that can exceed 40 °C, and variable rainfall most of which occurs during the summer. The mountain streams and pools are mainly ephemeral, although pools in some canyons may contain water year-round. The temporary nature of the freshwater habitats in the Sierra El Aguaje probably results in a cycle of local extinctions, population bottlenecks and recolonization events in the aquatic organisms in this region, which could lead to large population fluctuations and decreases in genetic variability. In addition, the desert landscape surrounding the Sierra El Aguaje potentially provides a formidable barrier to gene flow among aquatic organisms from neighboring mountain ranges, increasing the likelihood for genetic isolation and incipient speciation. Several plant and animal species, or subspecies, are known to be endemic to the Sierra El Aguaje [Felger, 1999; J.-P. Gallo-Reynoso, unpublished (available online at http://www.guaymas.gob.mx/ecologia/reserva.pdf)].

Leopard frogs inhabit the Sierra El Aguaje, but they have not been studied in detail, and virtually nothing is known of their taxonomy, physiological ecology or evolutionary history. Based on distributions of coastal leopard frogs given in Zaldívar-Riverón et al. (2004), *R. forreri*, although near the northern limit of its distribution (Fig. 1), would be expected to occur in the Sierra El Aguaje, and accordingly *R. forreri* has been listed in a species checklist of the fauna of this region (J.-P. Gallo-Reynoso, unpublished). However, in the formal description of *R. magnaocularis*, Frost and Bagn-

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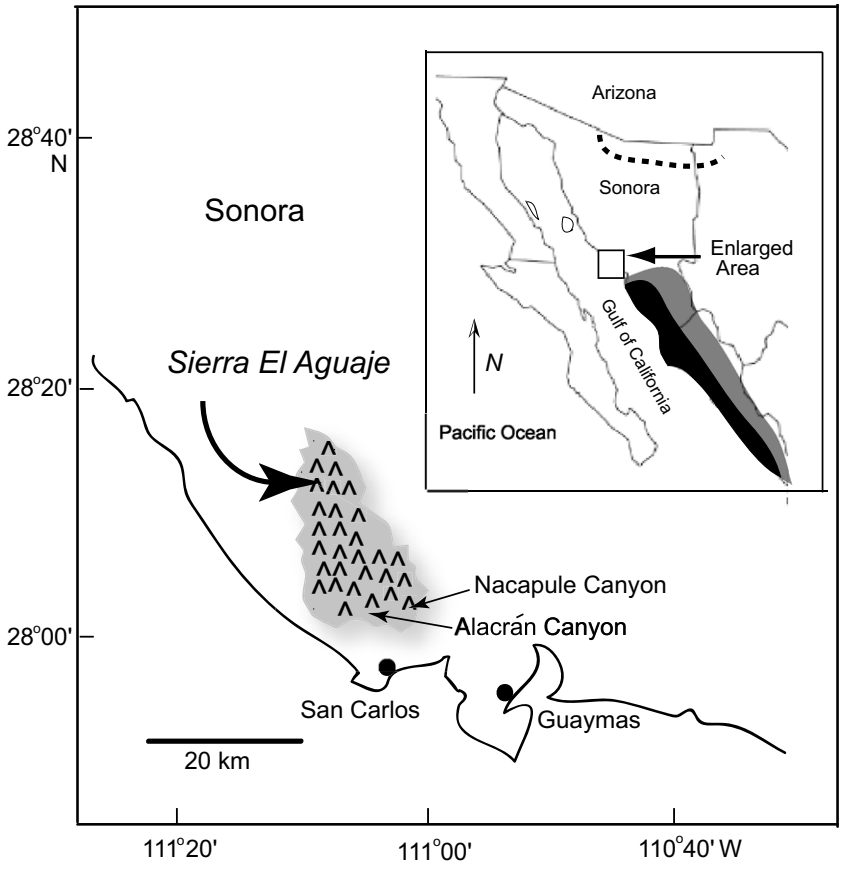


**Fig. 1.** Map showing the location of sampling sites (Nacapule and Alacrán Canyons) in the Sierra El Aguaje in southern Sonora, Mexico. Dotted line in the inset shows approximate southern distribution limits of *Rana yavapaiensis* in northern Sonora. Black shaded area (inset) shows where *R. magnaocularis* and *R. forreri* occur sympatrically in northwestern Mexico; grey shaded area shows additional range of *R. magnaocularis*.

ara (1976) noted that *R. forreri* and *R. magnaocularis* occurred sympatrically at several locations in southern Sonora, approximately 100 km SE of the Sierra El Aguaje (Fig. 1). Based on habitat preferences of *R. forreri* and *R. magnaocularis* (Frost and Bagnara, 1976), *R. magnaocularis* would be the more likely of the two species to inhabit the predominately xeric environment of the Sierra El Aguaje. Also, a third species, *R. yavapaiensis*, is known from northern Sonora and neighboring Arizona (Platz and Frost, 1984), but its southern distribution limit in Mexico is unclear. Thus, the possibility exists that it too might be found in the Sierra El Aguaje (M.J. Srdel, pers. comm.).

In the present study we used mitochondrial DNA (mtDNA) sequence data from the control region and 12S rRNA in a sample of leopard frogs from the Sierra El Aguaje, together with available sequences from leopard frogs deposited in GenBank, to infer taxonomic identity and provide phylogenetic hypotheses for relationships with other selected members of the *R. pipiens* complex.

## 2. Materials and methods

### 2.1. Sample collection

Tissue samples consisting of toe clips (adults) or tail clips (larvae) were taken from a total of 15 frogs collected from pools at two localities in the Sierra El Aguaje, near San Carlos, Sonora, Mexico on 3–5 March, 2005 (Fig. 1); samples were immediately placed in 95% ethanol. The pools, located in Nacapule Canyon (28°01′N, 111°03′W) and Alacrán Canyon (28°01′N, 111°06′W), were ~5 km apart with no drainages connecting the two. Ten samples were obtained from Nacapule Canyon and five were from Alacrán Canyon.

### 2.2. DNA extraction and amplification

Total genomic DNA was extracted from tissue samples using the DNeasy™ (QIAGEN Inc., Valencia, CA) protocol. The polymerase chain reaction (PCR) was used to amplify the control region using primers CytbA-L and ControlP-H; primers 12SJ-L and 12SK-H were used to amplify 12S rRNA. Descriptions of both pairs of primers are given in Goebel et al. (1999).

Forward and reverse sequencing reactions were performed on an Applied Biosystems (Foster City, CA) ABI 3730XL DNA sequencer at the DNA Sequencing Facility, University of Arizona, using the amplifying primers. Alignments were performed in ClustalX 1.81 (Thompson et al., 1997) followed by manual editing.

Calculations of genetic distances (uncorrected *p*-distances) among leopard frog sequences and base composition analyses were carried out in MEGA version 3.1 (Kumar et al., 2004). The 12S rRNA and control region sequences for leopard frogs from the Sierra El Aguaje (hereafter referred to as *Rana* sp. Aguaje) have been deposited in GenBank (Accession Nos. EU728669 and EU728670).

### 2.3. Phylogenetic analyses

Phylogenetic relationships among leopard frogs were assessed by maximum parsimony (MP), maximum likelihood (ML), and

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Bayesian inference for aligned control region and 12S rRNA sequences for *Rana* sp. Aguaje and representative sequences for leopard frogs available in GenBank (Table 1). The control region sequences listed in Table 1 were trimmed at both ends to yield a comparable 956 bp segment among species. The aligned 12S rRNA segment containing 825 bp was trimmed to 815 bp after removing ambiguous alignments at positions 354–363. We also present analyses of a shorter segment of 12S rRNA (405 bp) that was trimmed to 393 bp by deleting ambiguous alignments at positions 206–217. The matrices of aligned control region and 12S rRNA sequences used to generate the phylogenetic trees in this study are available in TreeBASE (Accession No. S2100).

The MP analyses were carried out in MEGA using the Max-mini branch-and-bound algorithm (815 bp data set) or the CNI heuristic search option (393 bp data set) with 100 random additions of sequences (Kumar et al., 2004). Gaps were coded as missing data. The ML analyses were carried out in DAMBE (Xia and Xie, 2001) using the nucleotide substitution models described below for the Bayesian analyses. Relative support for MP and ML tree topology was obtained by bootstrapping (Felsenstein, 1985) using 1000 pseudoreplicates. Bayesian methods were implemented in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). Bayesian analyses were run for 1,000,000 generations, sampled every 250th generation (4000 trees sampled), using the default random tree option to begin the analysis and applying the HKY nucleotide substitution model (nst = "2"; rates = "invgamma") for the control region (Jaeger et al., 2001), or the GTR model (nst = "6"; rates = "invgamma") for 12S rRNA (Zaldívar-Riverón et al., 2004). Log-likelihood values were calculated for four simultaneous MCMC chains (three hot and one cold). For each gene, we also carried out runs using the alternate nucleotide substitution model (GTR for the control region and HKY for 12S rRNA) and in both cases found similar clade support values and no differences in tree topologies. Clade support, expressed as posterior probabilities, was estimated utilizing a Markov chain Monte Carlo (MCMC) algorithm. For each gene, the first 20 trees were discarded from analysis (burnin = 20). *Rana pipiens* was used as the outgroup in all analyses.

## 3. Results and discussion

### 3.1. Sequence analysis

PCR of the control region yielded a 994 bp segment in 14 of 15 individuals of *Rana* sp. Aguaje from the two localities in the Sierra El Aguaje; a shorter segment of 892 bp was obtained for the remaining individual from Alacrán Canyon. For 12S rRNA, 814 bp were clearly resolved in 12 of the 15 individuals (poorly resolved sequences from 3 individuals from Nacapule Canyon were omitted from further analysis). Base composition was 35.6% T, 20.4% C, 33.9% A and 10.1% G for the control region ($N = 14$) and 24.2% T, 23.8% C, 31.9% A and 20.1% G for 12S rRNA ($N = 12$). Base composition of the 12S rRNA gene segment was identical to that reported by Zaldívar-Riverón et al. (2004) for the corresponding segment from a variety of species of Mexican leopard frogs. Both the control region and 12S rRNA of *Rana* sp. Aguaje contained just a single haplotype [i.e. haplotype diversity ($h$) = 0; nucleotide diversity ($\pi$) = 0]. The shorter 892 bp control region segment omitted from base composition analysis also showed no base substitutions.

Although our focus was not on population genetics, the complete lack of genetic variability in both gene segments of *Rana* sp. Aguaje is consistent with populations which periodically undergo severe bottlenecks, although other explanations, such as selective sweeps or populations historically located on the periphery of the species' range (Hoffman and Blouin, 2004b), cannot be ruled out. A complete lack of genetic diversity in the corresponding control region segment was also found for *R. onca* ($N = 9$) from seven localities in northwestern Arizona and southern Nevada (Jaeger et al., 2001). Sredl et al. (1997) has pointed out that populations of leopard frogs in the western USA are prone to large fluctuations, which is consistent with the above findings, as well as those of

**Table 1**
Species of leopard frogs (*Rana*) analyzed in this study, with collecting localities and GenBank Accession Numbers

| Species | Locality | GenBank Accession No. | |
|---|---|---|---|
| | | Control region | 12S rRNA |
| *R. berlandieri* | Brewester Co., Texas | AF343781 | |
| | Tamaulipas, Mexico | | AY115110 |
| *R. blairi* | San Miguel Co., New Mexico | AF343780 | |
| | Douglas Co., Kansas | | AY779237 |
| *R. brownorum* | Chiapas, Mexico | | AY115120 |
| *R. chiricahuensis* | Chiricahua Mtns., Arizona | AF343783 | |
| | Greenlee Co., Arizona | | DQ283270 |
| | Apache Co., Arizona | | AY779226 |
| | Durango, Mexico | | AY779225 |
| *R. forreri* | Sonora, Mexico | | AY115123 |
| | Michoacán, Mexico | | AY115127 |
| | Oaxaca, Mexico | | AY115129 |
| | Oaxaca, Mexico | | AY115130 |
| *R. magnaocularis* | Huajimic, Nayarit, Mexico | | AY115131 |
| | near Nuri, Sonora, Mexico | | AY779239 |
| *R. onca* | Clark Co., Nevada | AF343776 | |
| | Clark Co., Nevada | | AY779249 |
| *R. pipiens* | Lincoln Co., Nevada | AF343782 | |
| | Michigan | | DQ283123 |
| | Ottawa Co., Ohio | | AY779221 |
| *R. yavapaiensis* | Yavapai Co., Arizona | AF343777 | |
| | Pima Co., Arizona | | DQ283272 |
| | Greenlee Co., Arizona | | AY779240 |
| *Rana* sp. 1 | Michoacán, Mexico | | AY115135 |
| *Rana* sp. Papagayo | Guerrero, Mexico | | AY115134 |
| *Rana* sp. 7 | Jalisco, Mexico | | AY779241 |
| *Rana* sp. 8 | Puebla, Mexico | | AY779248 |
| *Rana* sp. Aguaje | Sierra El Aguaje, Sonora, Mexico | EU728670 | EU728669 |

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Hoffman and Blouin (2004a) who found reduced genetic diversity in western populations of *R. pipiens* compared to those from the eastern USA.

### 3.2. Phylogenetic analyses

Published control region sequences were available for only six leopard frog species from southwestern USA and northwestern Mexico for comparison with *Rana* sp. Aguaje (Table 1). The MP, ML and Bayesian phylogenetic analyses yielded identical trees (not shown) which indicated that *Rana* sp. Aguaje clustered in a highly-supported (100%) clade with *R. yavapaiensis* and *R. onca*. Genetic distance between *Rana* sp. Aguaje and the *R. yavapaiensis*/*R. onca* species pair was ~11%. *Rana berlandieri* and *R. blairi* clustered in a separate clade, also highly supported. Finally, *R. chiricahuensis* resolved as the basal lineage. Other than the inclusion of *Rana* sp. Aguaje, the only difference between our trees and those of Jaeger et al. (2001) was the interchanged positions of *R. chiricahuensis* and *R. pipiens* as their trees were rooted with the former species. Because of the limited number of taxa, our control region trees provided minimum resolution and thus are not presented, but nonetheless they strongly suggested that *Rana* sp. Aguaje, although related to *R. yavapaiensis*, represents a separate lineage.

Phylogenetic analyses of the 12S rRNA data set were more informative owing to the larger number of GenBank sequences available for comparison (Table 1), most of which were from the study on Mexican leopard frogs by Zaldívar-Riverón et al. (2004). Because of the disparity in number of species represented in the 12S rRNA and control region data sets, in addition to the fact that both genes were not sequenced in the same individuals (except for *Rana* sp. Aguaje), we did not conduct combined analyses. For the 12S rRNA analyses we used a representative subset of species and evolutionary lineages reported in Zaldívar-Riverón et al. (2004), which included *R. magnaocularis* from Huajimic, Nayarit, (~1000 km SE of the Sierra El Aguaje) and two undescribed lineages closely related to *R. magnaocularis* (*Rana* sp. Papagayo and *Rana* sp. 1), four lineages of *R. forreri*, and single representatives of *R. berlandieri* and *R. brownorum*. 12S rRNA sequences for two species not analyzed by Zaldívar-Riverón et al. (2004), *R. yavapaiensis* and *R. chiricahuensis* (Frost et al., 2006), were also included. All of these species, except for *R. chiricahuensis* and *R. pipiens*, are members of the *R. berlandieri* subgroup, or *Scurrilirana* subclade, of the *R. pipiens* group (*Pantherana* clade) (Hillis and Wilcox, 2005). The MP, ML and Bayesian trees were concordant and showed that *R. yavapaiensis* clustered in a clade that included *Rana* sp. Aguaje, in agreement with the control region trees, and *R. magnaocularis* from Nayarit, Mexico (Fig. 2A). *Rana* sp. Papagayo + *Rana* sp. 1 resolved as a sister clade to *R. yavapaiensis* + *R. magnaocularis* + *Rana* sp. Aguaje with strong support in the Bayesian tree (posterior probability = 0.97) but with poor support in the MP (54%) and ML (52%) trees. The corresponding clade (i.e. *R. magnaocularis* + *Rana* sp. Papagayo + *Rana* sp. 1) from Zaldívar-Riverón et al. (2004) also was more highly supported in Bayesian analyses than those using maximum parsimony. Overall, the inclusion of *R. yavapaiensis* and *Rana* sp. Aguaje in our trees resulted in only a small change in the topology of the MP and Bayesian trees reported by Zaldívar-Riverón et al. (2004). Those authors showed *Rana* sp. Papagayo as sister to *R. magnaocularis*, with *Rana* sp. 1 as basal.

Genetic distance between *Rana* sp. Aguaje and *R. magnaocularis* from Nayarit based on the 12S rRNA sequences was small (1.8%) suggesting that the two lineages are closely related. Based on this information alone, however, it is unclear whether they should be considered conspecific. Genetic distance between the two lineages of *R. forreri* from Oaxaca, which may be distinct at the species level, is also 1.8% (Zaldívar-Riverón et al., 2004). Genetic distance between *Rana* sp. Aguaje and *R. yavapaiensis* was 2.9%. The four lineages of *R. forreri* clustered in a highly-supported clade in all our analyses (Fig. 2A), in agreement with the findings of Zaldívar-Riverón et al. (2004). Genetic distance between *Rana* sp. Aguaje and *R. forreri* from southern Sonora was 5.6%. Overall, genetic distances between *Rana* sp. Aguaje and the four representative of the *R. forreri* complex ranged from 5.4–6.9%.

We also analyzed additional partial segments of the 12S rRNA gene for several species and undescribed evolutionary lineages of leopard frogs from the mtDNA study of Hillis and Wilcox (2005) (Table 1). Hillis and Wilcox (2005) sequenced the region of the mitochondrial genome that included 12S rRNA through 16S rRNA. This included a 393 bp segment of the 12S rRNA gene (see Materials and methods) that overlapped our 815 bp data set. Although less informative because of the reduced number of characters, these additional sequences included another specimen identified as *R. magnaocularis* collected near Nuri, Sonora in the Sierra Madre Occidental, ~160 km E of the Sierra El Aguaje and in a region previously shown to contain *R. magnaocularis* (Frost and Bagnara, 1976), as well as two unidentified lineages from Mexico (*Rana* sp. 7 and 8) which clustered in a clade that included *R. magnaocularis*, *R. yavapaiensis* and *R. onca* (Hillis and Wilcox, 2005). The MP, ML and Bayesian phylogenetic analyses of the truncated data set were generally congruent, and again resolved a *Scurrilirana* clade which was well supported, especially in the Bayesian tree (Fig. 2B). In agreement with Fig. 2A, *Rana* sp. Aguaje resolved as sister to *R. magnaocularis* from Nayarit. Also in agreement with Fig. 2A, a clade containing the evolutionary lineages of *R. forreri* was obtained in all three analyses, but was most highly supported in the Bayesian tree. Unexpectedly, however, *R. magnaocularis* from Nuri, Sonora, resolved as sister to *R. blairi* from Kansas in the Bayesian tree, forming a clade distinct from the one containing *R. magnaocularis* from Nayarit. The *R. magnaocularis* (Sonora) + *R. blairi* clade, however, was not supported in the MP and ML analyses. There were 49 nucleotide substitutions in the 393 bp segment between the two lineages of *R. magnaocularis*, resulting in a genetic distance of 12.5%. Because several of the relationships shown in Fig. 2A were also recovered using the truncated data set, we suggest that the resolution of this data set, although not optimum, is sufficiently strong to suggest that cryptic but genetically distinct lineages of *R. magnaocularis* may occur in western Mexico. We cannot, however, rule out the possibility of specimen misidentification or mislabeling of the GenBank sequence for *R. magnaocularis* from Sonora.

Fig. 2B also shows that *Rana* sp. 1 from Michoacán (Zaldívar-Riverón et al., 2004) and *Rana* sp. 7 from Jalisco (Hillis and Wilcox, 2005) resolved as sister lineages in a clade that was highly-supported in the Bayesian and ML trees. These two unidentified lineages are very closely related, and possibly represent the same species. Only three nucleotide differences out of 393 bp separated the two lineages (*p*-distance = 0.8%) which were collected from neighboring states in western Mexico (Contla, Jalisco and the Sierra Coalcomán, Michoacán) separated by ~120 km. Hillis and Wilcox (2005) showed that *Rana* sp. 7 was sister to *R. magnaocularis* from Nuri, Sonora, and also showed that this clade was sister to a clade that included *R. yavapaiensis* + *R. onca* + *Rana* sp. 8.

The uncertainty that exists in the taxonomy and phylogenetic relationships of Mexican leopard frogs is not surprising given that the evolution of these frogs evidently has been accompanied by little external morphological change. Frost (1982) commented that *R. berlandieri*, *R. brownorum* and *R. forreri*, previously considered subspecies of *R. berlandieri* but now accepted as valid species, showed overall morphological similarities and only minor differences in color pattern. Also, taxonomic determinations can be further confounded by the fact that more than one species of leopard frog can inhabit the same locality (Platz and Platz, 1973; Frost and Bagnara, 1976). Mitochondrial DNA analyses suggest that

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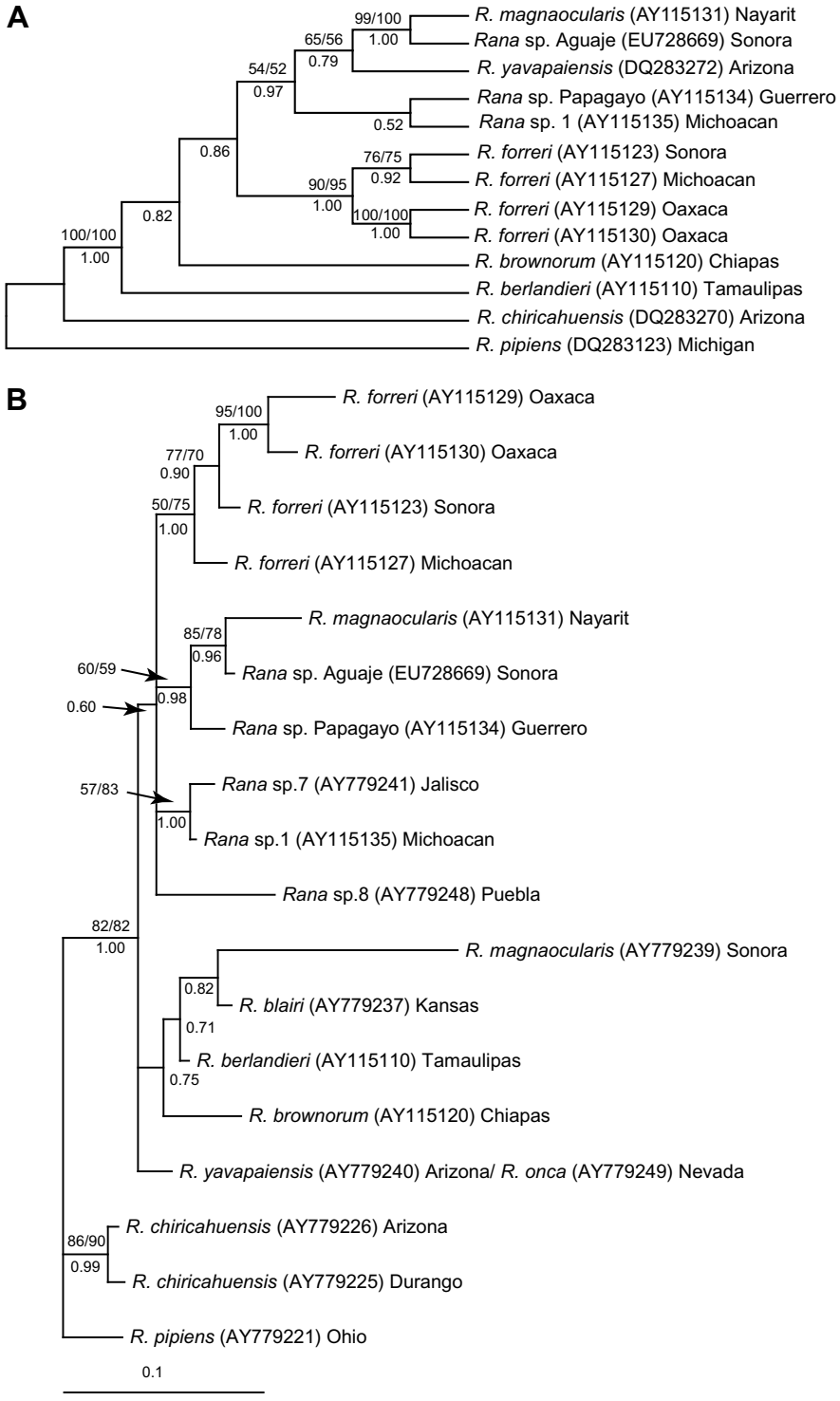


**Fig. 2.** (A) Most parsimonious tree (length = 243; CI = 0.700; RI = 0.629) obtained for species of leopard frogs ($N$ = 13) based on analysis of an 815 bp segment of the 12S rRNA gene (158 variable sites; 90 parsimony informative sites). *Rana pipiens* was the outgroup. Bootstrap support values >50% for the MP/ML trees are shown above the branches; posterior probabilities for the 50% majority rule Bayesian tree are shown below the branches; (B) Fifty-percent majority rule consensus tree showing relationships among species of leopard frogs based on Bayesian analysis of a 393 bp segment of the 12S rRNA gene. Relative position of clade support values on branches are the same as in Fig. 2A. Scale bar shows substitutions per site. *Rana pipiens* was the outgroup. Base composition was identical in the 393 bp segment of *R. yavapaiensis* and *R. onca*.

*R. magnaocularis* in western Mexico may represent a complex of cryptic species which have undergone substantial genetic divergence. In addition to the large genetic divergence noted between populations of *R. magnaocularis* from Sonora and Nayarit, Zaldívar-Riverón et al. (2004) stated that their unidentified *Rana* sp. 1 from Michoacán, Mexico (closely related to or conspecific with *Rana* sp. 7 of Hillis and Wilcox, 2005) was morphologically similar to *R. magnaocularis*, and showed that the two lineages resolved in the same clade, but that genetic distance among the two lineages was relatively large ($p$-distance = 6.4%). Mitochondrial DNA studies also suggest that the unidentified *Rana* sp. Papagayo is closely related to *R. magnaocularis* and *R. yavapaiensis* (Zaldívar-Riverón

et al., 2004; present study). In problematic taxa, such as the *R. pipiens* complex, where morphological characters often provide poor resolution, the use of mtDNA data to help establish species boundaries, although controversial, can be especially useful (Wiens and Penkrot, 2002). It is clear that additional studies, including molecular analyses, laboratory research on hybridization, and analyses of vocalizations and behavior are needed to clarify the taxonomic identity and phylogenetic relationships of leopard frogs from Mesoamerica.

In summary, the results of the present mtDNA study suggest that *Rana* sp. Aguaje belongs to the *R. berlandieri* subgroup, or *Sierrilirana* clade, of the *R. pipiens* group (Hillis and Wilcox, 2005), and is most closely related to a lineage of leopards frogs from Nayarit, Mexico identified as *R. magnaocularis* (Zaldívar-Riverón et al., 2004). We also have provided further evidence that *R. magnaocularis* and *R. yavapaiensis* are close relatives, as previously suggested from both morphological (Platz and Frost, 1984), allozyme (Hillis et al., 1983) and mtDNA analyses (Hillis and Wilcox, 2005).

## Note added in proof

Based on analyses of 12S rRNA sequences (814 bp), we have recently discovered that the *Rana* sp. Aguaje lineage ($N = 6$) also occurs at two sites near Nuri, Sonora and thus is not restricted to the Sierra El Aguaje.

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