

Phylogeny of the Mexican coastal leopard frogs of the *Rana berlandieri* group based on mtDNA sequences

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Abstract

Phylogenetic relationships among specimens from 25 different locations for the six Mexican coastal leopard frog species of the *Rana berlandieri* species group were investigated using 797 bp of the mitochondrial 12S rDNA gene. Relationships among the haplotypes obtained were recovered using maximum parsimony and Bayesian analyses. Most of the clades recovered by both tree building methods are strongly supported, but conflicting clades recovered by each analysis are generally poorly supported. Both analyses reject the previously proposed subgroupings of the *R. berlandieri* species group. Based on the strongly supported relationships, genetic differentiation, and geographic distribution of the haplotypes examined, nine independent lineages appear to comprise the group of study. However, confirmation of the new proposed lineages will require further analyses based on other genetic markers and additional samples that cover their entire geographic distribution. Concordance was noted between Miocene–Pliocene geological and climatic events in Mexico and the relationships recovered among the lineages proposed and their geographic distribution.

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1. Introduction

Leopard frogs (genus *Rana*) constitute a conspicuous component of the herpetofauna of the coastal plains and adjacent lowlands of Middle America. Currently, only three species of these frogs (referred to hereinafter as coastal leopard frogs for simplicity) are recognized in the Mexican territory: *Rana berlandieri*, *Rana brownorum*, and *Rana forreri* (Flores-Villela, 1993; Hillis, 1988). Additionally, three species of coastal leopard frogs have been informally recognized by Hillis et al. (1983), but are still undescribed, and it is possible that yet other species remain to be discovered (Hillis, 1988). The coastal leopard frogs comprise a clade deeply nested in the *Rana*

pipiens complex (Hillis et al., 1983), a monophyletic group of over 20 species distributed throughout almost all of the biotic regions from central and eastern United States south and east to central Panama (Hillis, 1988; Hillis et al., 1983). Species of this complex are characterized by possessing a remarkably conserved external morphology, a reason why they have been historically difficult to delimit (Hillis, 1988; Moore, 1975).

On the basis of allozyme data, Hillis et al. (1983) performed the only phylogenetic analysis available of the *R. pipiens* complex. In their phylogenetic hypothesis, the 23 examined species of the complex formed two broadly sympatric major clades (the alpha and beta divisions), each containing two species groups (one North American and another one Middle American) with parapatric distributions between their members (Fig. 1). With a total of 13 species, the *R. berlandieri* species group of the beta division was the most diverse of these four groups, being further divided by the authors into three small subgroups. One of these subgroups was composed of four species from the foothills of the Sierra

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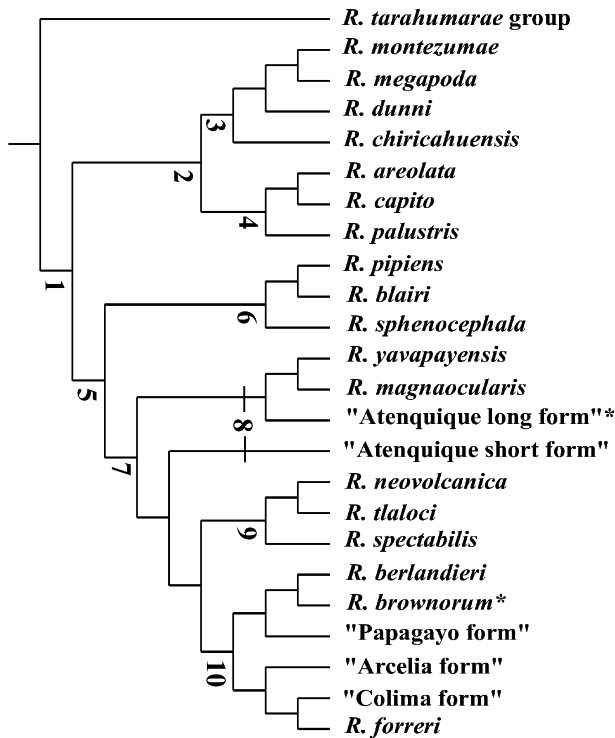


Fig. 1. Hypothesis on the relationships among the examined species of the *Rana pipiens* complex (modified after Hillis et al., 1983). 1, *R. pipiens* complex; 2, alfa division; 3, *R. montezumae* species group (Middle American); 4, *R. areolata* species group (North American); 5, beta division; 6, *R. pipiens* species group (North American); 7, *R. berlandieri* species group (Middle American); 8, "Foot Hills of the Sierra Madre Occidental" subgroup; 9, "Mexican Plateau" (Transvolcanic Belt) subgroup; and 10, Mexican coastal subgroup. *, Relationships based only on morphology.

Madre Occidental, another one contained three species from the Transvolcanic Belt, and the remaining one was composed of six species from the Mexican coastal plains and adjacent lowlands (the coastal clade). Of the species in the latter clade, three (the "Arcelia," "Colima," and "Papagayo" forms) were only informally recognized in this study. Furthermore, although not included, Hillis et al. (1983) suggested that two additional species from Central America, *Rana miadis* and *Rana taylori*, also probably belong to the coastal clade.

Although Hillis et al. (1983) provided a preliminary understanding of the systematics of the *R. pipiens* complex, the diversity, geographic distribution, and hence evolutionary relationships of the coastal leopard frogs have not been thoroughly investigated. One major problem is that *R. brownorum* was included in Hillis et al.'s (1983) analysis only on the basis of morphological data. This taxon was originally described from the Caribbean lowlands of southeastern Mexico (41 miles west of Xicalango, Campeche) as a subspecies of *R. berlandieri* (Sanders, 1973); however, it was subsequently elevated to full species status by Hillis (1981) but without any justification. Thus, its taxonomic status is

uncertain. Also, the inland distribution of *R. brownorum* in the Peninsula de Yucatan and rest of southeastern Mexico is not adequately known (Lee, 1999). Specimens deposited in the Museo de Zoología of the Facultad de Ciencias, Universidad Nacional Autónoma de México from different localities in southeastern Mexico east of the Isthmus of Tehuantepec, including localities in the mountain ranges of Chiapas, are morphologically similar to *R. brownorum* (unpublished information). However, because they came from localities distant from the known distribution of *R. brownorum*, and located in different physiographic regions, their assignment to this taxon remains in doubt.

After Hillis et al.'s (1983) analysis, it became evident that *R. forreri*, previously considered to occur along the Pacific coastal plain and adjacent lowlands of Mexico and Central America, actually represented a composite taxon. In addition, specimens from the type locality of *R. forreri* (Presidio, Sinaloa) and nearby localities in northwestern Mexico, as well as specimens from the localities recorded for the undescribed species identified by Hillis et al. (1983), appear to differ from specimens from other putative populations of *R. forreri* in some other localities in Mexico (unpublished information).

In this study, we examined the variation in the mitochondrial (mt) DNA sequence of a fragment of the 12S ribosomal DNA (rDNA) gene among individuals from 25 different localities along the Atlantic and Pacific coasts and adjacent lowlands of Mexico, representing the six species of the coastal clade of the *R. berlandieri* species group (*sensu* Hillis et al., 1983), with an emphasis on *R. brownorum* and *R. forreri*. We used this variation to investigate the number of evolutionary lineages in this group and their phylogenetic relationships using maximum parsimony and Bayesian analyses. Because Hillis et al. (1983) analyzed their allozyme data without the aid of any computer software, we also reanalyzed this information in order to compare it with our data.

2. Materials and methods

2.1. Specimen information

Sequences were obtained from 25 specimens assigned to the six species belonging to the *R. berlandieri* Mexican coastal clade of Hillis et al. (1983). Eleven of these specimens were assigned to *R. forreri* and eight to *R. brownorum*. All of the specimens were collected from different localities along the Pacific, Gulf of Mexico, and Caribbean coastal plains and adjacent lowlands of Mexico, as well from some localities situated in the highlands of Chiapas (Fig. 2). Monophyly of the Mexican coastal leopard frogs was tested by including in the ingroup sequences of three species belonging to the other two subgroupings of the *R. berlandieri* species

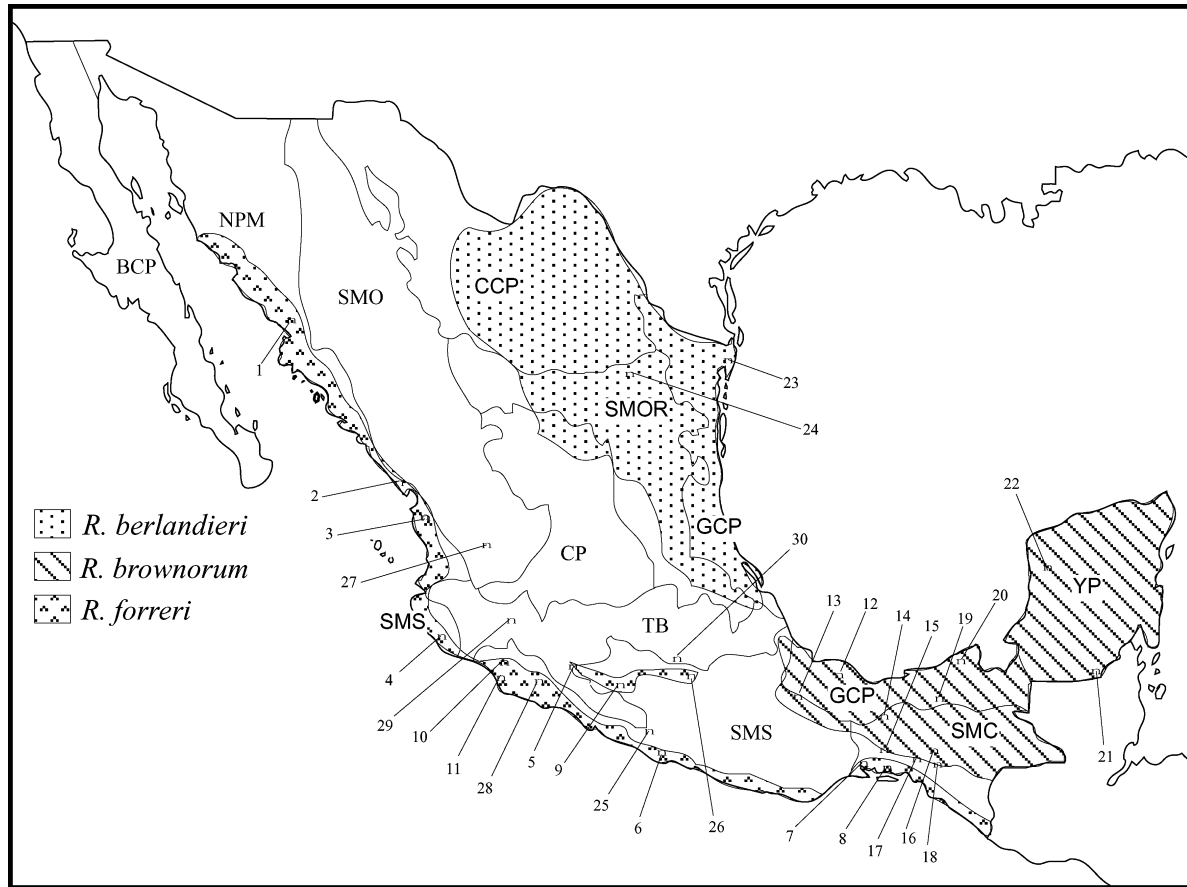


Fig. 2. Distribution in Mexico of the described species belonging to the Mexican coastal clade *sensu* Hillis et al. (1983) [distribution of *Rana berlandieri* and *R. brownorum* based on museum specimens (Zaldívar-Riverón, unpublished data) and literature records; distribution of *R. forreri* as known before Hillis et al. (1983)]. Black dots represent the localities sampled for the species examined in this study; numbers correspond to the species assignment listed in Table 1. Mexican physiographic provinces: BCP, Baja California Province; NPM, Northwest Plains and Mountain Ranges; SMO, Sierra Madre Occidental; CCP, Chihuahua and Coahuila Plateaus and Mountain Ranges; SMOR, Sierra Madre Oriental; GCP, Gulf Coastal Plain; CP, Central Plateau; TB, Transvolcanic Belt; SMS, Sierra Madre del Sur; SMC, Sierra Madre de Chiapas; and YP, Yucatan Platform.

group (*Rana neovolcanica*, *Rana spectabilis*, and *Rana magnaocularis*), and also the sequence of an undescribed species morphologically similar to *R. magnaocularis* (hereinafter referred to as *R. sp. 1*). Additionally, a sequence of *Rana montezumae* obtained by us and another one of *R. pipiens* taken from an earlier study (GenBank Accession No. Y10945; Feller and Hedges, 1998) were employed as outgroups. These latter species are members of the Mesoamerican *R. montezumae* and the North American *R. pipiens* species groups, respectively. Sampling for most of the described species included specimens from their type locality or a nearby site. For the undescribed species mentioned by Hillis et al. (1983) (hereinafter named as *R. sp. Arcelia*, *R. sp. Colima*, and *R. sp. Papagayo*), specimens were collected on the basis of their recorded collection sites. Voucher specimens for this study are deposited in the Museo de Zoología “Alfonso L. Herrera,” Facultad de Ciencias, Universidad Nacional Autónoma de México (MZFC). Specific designations, localities, voucher numbers, and GenBank accession numbers of the examined specimens are given

in Table 1. The Mexican physiographic provinces and subprovinces referred are those defined by Ferrusquía-Villafranca (1998).

2.2. Laboratory protocols

Total genomic DNA was extracted from ~100 mg of liver tissue following the standard phenol/chloroform extraction protocol given by Hillis et al. (1996). The amplified product was an approximately 860 bp fragment of the 12S rDNA gene. This gene was selected because it has been successfully used to infer phylogenetic relationships for other closely related species of ranids (e.g., Dawood et al., 2002; Emerson et al., 2000; Sumida et al., 2000). The following primers given by Goebel et al. (1999) were used for amplification and sequencing reactions (5′–3′): 12SJ-L: AAAGRTTGG TCCTRRSCTT; 12SK-H: TCCRGTAYRCTTACCDT GTTACGA. The PCR program for amplifications had a first cycle at 96 °C for 2 min, 50 °C for 45 s, and 72 °C for 2 min, followed by 39 cycles at 94 °C for 30 s, 50 °C

Table 1

List numbers, specific designations, localities, and voucher and GenBank numbers of the examined specimens in this study

No.	Species	Locality	Voucher Nos.	GenBank Accession Nos.
1	<i>Rana forreri</i> 1	Estación Don, Sonora	MZFC 14186	AY115123
2	<i>Rana forreri</i> 2	Río Presidio, 21 km E Mazatlán, Sinaloa	MZFC 14187	AY115124
3	<i>Rana forreri</i> 3	Ruiz, Río San Pedro, Nayarit	MZFC 13047	AY115125
4	<i>Rana forreri</i> 4	Chamela, Jalisco	NA	AY115126
5	<i>Rana forreri</i> 5	S El Caracol, Nuevo Urecho, Michoacán	MZFC 12805	AY115127
6	<i>Rana forreri</i> 6	Laguna Tres Palos, Acapulco, Guerrero	MZFC 14198	AY115128
7	<i>Rana forreri</i> 7	2 km SW Tehuantepec, Oaxaca	MZFC12875	AY115129
8	<i>Rana forreri</i> 8	5 km N Zanatepec, Oaxaca	MZFC HRG 170	AY115130
9	<i>Rana</i> sp. Arcelia	20 km E Arcelia, Guerrero	MZFC 14189	AY115109
10	<i>Rana</i> sp. Colima 1	Coquimatlán, Colima	MZFC 14202	AY115136
11	<i>Rana</i> sp. Colima 2	La Parotita, Colima	MZFC 14200	AY115137
12	<i>Rana brownorum</i> 1	Estación Biológica Los Tuxtlas, Veracruz	MZFC 14191	AY115112
13	<i>Rana brownorum</i> 2	Paso Canoa, Oaxaca	MZFC VLR 5012	AY115115
14	<i>Rana brownorum</i> 3	La Gringa, mpio. of Santa María Chimalapa, Oaxaca	MZFC 14193	AY115116
15	<i>Rana brownorum</i> 4	San Antonio Chimalapa, Oaxaca	MZFC 12895	AY115117
16	<i>Rana brownorum</i> 5	San Fernando, Chiapas	MZFC 14167	AY115118
17	<i>Rana brownorum</i> 6	Parque Laguna Bélgica, mpio. of Ocozocuahtla, Chiapas	MZFC 14169	AY115119
18	<i>Rana brownorum</i> 7	Tuxtla Gutierrez, Chiapas	MZFC 12196	AY115120
19	<i>Rana brownorum</i> 8	Pozo Gualas, km 39.5 Villahermosa-Frontera, Tabasco	MZFC 14199	AY115121
20	<i>Rana brownorum</i> 9	Quintín Arauz, Pantanos de Centla, Tabasco	MZFC 14188	AY115122
21	<i>Rana brownorum</i> 10	La Unión, Quintana Roo	MZFC 14192	AY115113
22	<i>Rana brownorum</i> 11	Cenote Noh-Choncunchey, mpio. of Celestún, Yucatan	MZFC VLR 2012	AY115114
23	<i>Rana berlandieri</i> 1	Faro de Playa Bagdad, mpio. of Matamoros, Tamaulipas	MZFC 13727	AY115110
24	<i>Rana berlandieri</i> 2	Salto Cola de caballo, Nuevo León	MZFC 14246	AY115111
25	<i>Rana</i> sp. Papagayo	1 km SW Plan de Lima, Río Papagayo, Guerrero	MZFC 14190	AY115134
26	<i>Rana spectabilis</i>	2 km al N Oacalco, Morelos	MZFC 14194	AY115138
27	<i>Rana magnaocularis</i>	Huajimic, Nayarit	MZFC 13091	AY115131
28	<i>Rana</i> sp. 1	Sierra Coalcomán, Michoacán	MZFC 13028	AY115135
29	<i>Rana neovolcanica</i>	Rancho Club Tapalpa, mpio. of Tapalpa, Jalisco	MZFC 13071	AY115133
30	<i>Rana montezumae</i>	San Pedro Tlaltizapan, Estado de México	MZFC D82	AY115132

All localities are from Mexico.

for 45 s, 72 °C for 1.5 min, with a 10 min final extension. PCR were carried out in a 25 µl volume consisting of 2 µl of template DNA, 2 µl of each primer (5 µM), 0.5 µl dNTPs (0.8 mM), 2.5 µl of 10× PCR buffer, 0.8 µl of MgCl₂, 0.2 µl of *Taq* DNA polymerase (5 U/µl), and 15 µl of H₂O. PCR products were purified using Qiagen's QIAquick Spin PCR Purification Kit (Qiagen) and then sequenced employing the dideoxy terminator cycle sequencing (Applied Biosystems) and an ABI 373A automated DNA sequencer.

2.3. Phylogenetic analysis

Sequences were aligned using the multiple sequence alignment program Clustal W (Thompson et al., 1994) with the following parameters: opening gap cost = 10, gap extension cost = 5, delay divergent sequences = 40%, and transitions = unweighted. Regions that appeared to be of uncertain alignment were excluded. These ambiguities were located in the loop regions between the stems 17 and 18, 18' and 17', and 34 and 34' of the secondary structure model for the mammalian 12S rDNA given by Springer and Douzery (1996), and in the loop region between the stems 39' and 38' of the 12S third domain motif given by Hickson et al. (1996).

Base frequencies, pairwise uncorrected sequence divergences, and statistical analyses were obtained using the program PAUP* version 4.0b10 (Swofford, 2001). Phylogenetic signal in the data set was evaluated using the g_1 statistic (Hillis and Huelsenbeck, 1992) with 10,000 randomly generated trees. Two methods of phylogenetic reconstruction were used. First, an equally weighted maximum parsimony analysis using PAUP* version 4.0b10 (Swofford, 2001) was performed with a branch and bound search, considering character states as unordered and gaps as missing data. Clade support was evaluated using nonparametric bootstrap with 1000 pseudoreplicates and employing a branch and bound search, with values $\geq 70\%$ considered as strongly supported (Hillis and Bull, 1993). Additionally, Bremer support values for all internal branches were estimated using TreeRot version 2 (Sorenson, 1999) with its defaults parameters (20 replicate heuristic searches with random addition of taxa for each constraint statement).

Bayesian analyses using Metropolis-coupled Markov chain Monte Carlo (MCMC) were performed using MrBayes 2.0 (Huelsenbeck and Ronquist, 2001). To decrease the chance of reaching apparent stationary on local optima, two separate analyses were conducted.

Each analysis consisted of four chains, random starting trees, and uniform prior distribution of parameters, and used the appropriate model of sequence evolution (GTR+I+ Γ) determined by Modeltest version 3.0 (Posada and Crandall, 1998). The chains were run for four million generations, sampling trees every 100 generations. Stationary was determined visually, burn-in trees discarded, and the remaining trees used to estimate Bayesian posterior probabilities. We considered that clades were significantly supported if they were present in $\geq 95\%$ of the sampled trees (Huelsenbeck and Ronquist, 2001; Wilcox et al., 2002).

The Wilcoxon signed-ranks (Felsenstein, 1985; Templeton, 1983) and the Shimodaira–Hasegawa (Shimodaira and Hasegawa, 1999) tests were executed to test for significant differences of alternative topologies in the maximum parsimony and Bayesian analyses, respectively. Three alternative hypotheses were employed, which constrained the following groups as monophyletic: (1) species belonging to the Mexican coastal clade *sensu* Hillis et al. (1983), (2) the included species from the Transvolcanic Belt province, *R. spectabilis* and *R. neovolcanica*; and (3) the haplotypes herein assigned to *R. forreri*. The Wilcoxon signed-ranks tests were applied as one-tailed probability. In cases that the large-sample approximation was less than 25, the critical values were obtained from the table given by Siegel (1956). The Shimodaira–Hasegawa tests were executed using a full likelihood optimization and 1000 bootstrap replicates. The maximum parsimony and Bayesian analyses performed for constructing the alternative hypotheses used the same search strategies and parameters as above, except for the number of run generations in the Bayesian analyses, which was of 1 million.

2.4. Phylogenetic analysis of allozymic data

Hillis et al.'s (1983) published most parsimonious tree for allozyme data was based on 50 gene loci and 283 different electromorphs, the latter coded as character states. However, this tree was constructed manually and without the aid of any computer software. Therefore, we reanalyzed the same data with PAUP* version 4.0b10 (Swofford, 2001), using an equally weighted maximum parsimony analysis with a branch and bound search and the absence–presence coding method for allozyme data as implemented by Macey et al. (1999, 2001). If two electromorphs were present for a locus in a single terminal taxon, the locus was coded as polymorphic. Statistical comparisons between the resulting hypothesis derived from this data set and the alternative hypothesis mentioned above were not performed because it provided few phylogenetically informative characters. The data matrices of the allozyme and DNA sequence data can be found in <http://www.ibiologia.unam.mx/directorio/LeonRegagnon.htm>.

3. Results

3.1. Sequences

The length of the 12S rDNA gene fragment varied from 836 to 862 bp among the 31 examined specimens. Exclusion of sites with missing data at both extremes of the alignment and of ambiguously aligned regions resulted in 797 aligned positions. Twenty-eight haplotypes were found, 22 corresponding to the species of the Mexican coastal clade, four to the other included members of the *R. berlandieri* species group, and two to the outgroups. Identical haplotypes were found in *R. brownorum* 1 and 6, *R. brownorum* 2 and 10, and *R. berlandieri* 1 and 2. Only one sequence from individuals with identical haplotypes was included in the analyses. Base composition was slightly biased negatively against guanine (G=0.20, A=0.32, C=0.24, and T=0.24), which has been observed for the mitochondrial genome of other anuran groups (e.g., Dawood et al., 2002; Macey et al., 1998; Mulcahy and Mendelson, 2000).

Genetic distances between haplotypes ranged from 5.82 to 9.97% between the ingroup and outgroups and from 0.13 to 7.68% within the ingroup. In some cases, sequences from specimens assigned to *R. forreri* and *R. brownorum* were less similar to each other (0.13–5.12% and 0.13–2.77%, respectively) than they were to sequences from other ingroup species (0.78–7.68%). Moreover, there appears to be a relationship between geographic distance and genetic divergence among specimens of each of the latter two taxa.

3.2. Phylogenetic analyses

The data set contains a significant phylogenetic signal ($g_1 = -0.4592$, $P < 0.01$; mean tree length \pm SD = 641.4 \pm 26.8, range 523–622; critical value obtained from Hillis and Huelsenbeck, 1992). The unweighted maximum parsimony analysis performed with the 28 haplotypes was based on 178 variable characters, 99 of which were parsimony informative. Fourteen equally most parsimonious trees (length = 315, CI excluding uninformative characters = 0.533, RI = 0.8) were obtained. The burn-in in the two Bayesian analyses performed occurred after 12,000 and 15,000 generations. The mean lnL score of these analyses for all the trees sampled at stationary were of -2928.091 and -2928.114, respectively. The topologies of the 50% majority rule consensus trees derived from these two analyses are identical.

The consensus trees recovered by the maximum parsimony (Fig. 3a) and Bayesian (Fig. 3b) analyses are considerably similar. Most of the clades recovered by both tree building methods are strongly supported by bootstrap, Bremer support, and Bayesian posterior probabilities values; conflicting clades are generally poorly supported. However, three clades of the maxi-

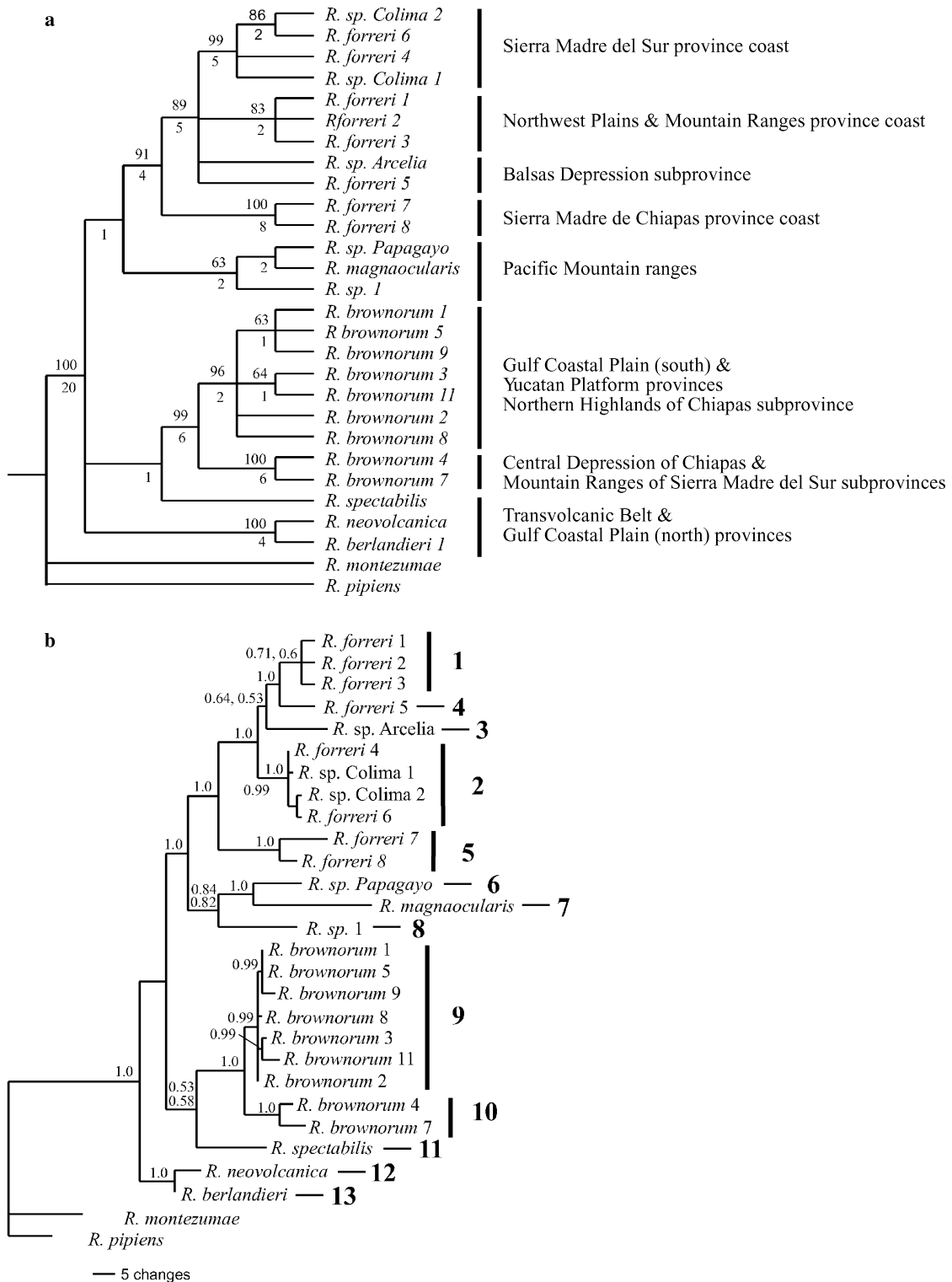


Fig. 3. Phylogenetic trees for the haplotypes found based on the examined fragment of the mtDNA 12S rDNA gene. (a) Strict consensus of the 14 most parsimonious trees showing the geographic distribution of the specimens examined. Bootstrap values for the nodes retained by more than 50% of bootstrap pseudoreplicates and Bremer support values are presented above and below branches, respectively. (b) Fifty percent majority rule consensus of the trees recovered in two Bayesian analyses using a 4-million generation Metropolis-coupled Markov chain Monte Carlo analysis. Mean branch lengths for the resolved clades correspond to one of the Bayesian analyses performed. Clade credibility values bigger than 50% are above branches (only one value is indicated when it was the same in both analyses). Numbers on the right of the terminal taxa represent the different haplotype groups identified in this study (see text).

maximum parsimony consensus tree, one with *R. forreri* 1–3, another one containing *R. sp. Colima* 2 and *R. forreri* 6, and the other one with all *R. brownorum* haplotypes except *R. brownorum* 4 and 7, are strongly supported by bootstrap values but have only a Bremer support = 2. Additionally, three clades are significantly supported by Bayesian posterior probabilities but are weakly supported by bootstrap values and have a Bremer support = 1 in the maximum parsimony analysis. Two of these clades are nested in a major clade with most of the *R. brownorum* haplotypes, whereas the third one contains all the haplotypes from the Pacific region.

The consensus trees of the maximum parsimony and Bayesian analyses present three similar major clades. In one of these, *R. neovolcanica* is strongly supported as the sister species of *R. berlandieri* (bootstrap = 100; Bremer support = 4; Bayesian posterior probability = 1.0). In a second major clade, the monophyly of all of the specimens assigned to *R. brownorum* is strongly supported (bootstrap = 99; Bremer support = 6; Bayesian posterior probability = 1.0). Within this clade, *R. brownorum* 4 + *R. brownorum* 7 (bootstrap = 100; Bremer support = 6; Bayesian posterior probability = 1.0), which come from two subprovinces of the Sierra Madre de Chiapas province (the Mountain Ranges of the Sierra Madre de Chiapas and the Central Depression of Chiapas, respectively), comprise the sister group of a clade composed of the haplotypes from the Gulf Coastal Plain and the Yucatan Platform provinces and a third Sierra Madre de Chiapas subprovince (the Northern Highlands of Chiapas) (bootstrap = 96; Bremer support = 2; Bayesian posterior probability = 1.0). The third major

clade, which contains all the haplotypes from the Pacific region, is supported by low bootstrap and Bremer support values (46 and 1, respectively) but has a Bayesian posterior probability of 1.0. In this clade, haplotypes from the mountain ranges adjacent to the Pacific coastal plain (the Sierra Madre Occidental and Sierra Madre del Sur) form a weakly supported subclade (bootstrap = 63; Bremer support = 2; Bayesian posterior probability = 0.82) exclusive of a well supported subclade of different Pacific coastal regions and the Balsas Depression subprovince (bootstrap = 91; Bremer support = 4; Bayesian posterior probability = 1.0).

The results of the Wilcoxon signed-ranks and the Shimodaira–Hasegawa tests indicate that the relationships recovered in our analyses give significantly better explanations than the three different alternative phylogenetic hypotheses tested (Table 2).

Our analysis of the allozyme data presented by Hillis et al. (1983) contained 283 characters, 91 of which were parsimoniously informative. The analysis resulted in 10 most parsimonious trees with length = 303, CI (excluding uninformative characters) = 0.461, and RI = 0.659. The strict consensus of these trees (Fig. 4) is poorly resolved, with few strongly supported clades, and differs in several relationships with respect to the phylogeny proposed by Hillis et al. (1983). Some notable differences are that, although the *R. berlandieri* group is recovered as monophyletic, this clade is weakly supported (bootstrap = 60; Bremer support = 2), and the Mexican coastal clade and the other two clusters of species proposed by Hillis et al. (1983) for this group were not recovered or were weakly supported.

Table 2
Results of the Wilcoxon signed-ranks and the Shimodaira–Hasegawa tests

Wilcoxon signed-ranks tests ^a					
Alternative hypotheses	MPT ^b	<i>L</i> ^c	<i>N</i> ^d	<i>Z</i> ^e	<i>P</i>
Hillis et al.'s (1983) Mexican coastal clade	8	335	23–26	3.198–3.49	<i>P</i> < 0.001*
<i>R. spectabilis</i> and <i>R. neovolcanica</i> as sister species	2	323	16	–	<i>P</i> < 0.002*
A clade with the specimens assigned to <i>R. forreri</i>	1	329	21	–	<i>P</i> < 0.003*
Shimodaira–Hasegawa tests ^f					
Alternative hypotheses	–ln AH ^g		Diff. –ln ^h		<i>P</i>
Hillis et al.'s (1983) Mexican coastal clade	–3246.181		318.039		<i>P</i> < 0.0001*
<i>R. spectabilis</i> and <i>R. neovolcanica</i> as sister species	–3037.827		109.685		<i>P</i> < 0.001*
A clade with the specimens assigned to <i>R. forreri</i>	–3127.231		199.089		<i>P</i> < 0.001*

^a Comparing the 14 most parsimonious trees obtained in this study with those recovered in the alternative hypotheses.

^b Number of most parsimonious trees obtained in the alternative hypotheses.

^c Length of the most parsimonious tree(s) obtained in the alternative hypotheses.

^d Range of the number of characters differing in minimum numbers of changes on paired topologies.

^e Range of the normal approximations of the Wilcoxon signed-ranks test for *N* ≥ 25 [for *N* < 25 statistical significance was based in Table given by Siegel (1956)].

^f Comparing the 50% majority rule consensus tree recovered in one of the Bayesian analyses with the 50% majority rule consensus tree obtained in the alternative hypotheses.

^g Mean ln*L* score of the alternative hypotheses.

^h Difference in the mean ln*L* scores between the hypothesis obtained in this study and the alternative hypotheses.

* Significant difference between the hypotheses obtained in this study and the alternative hypotheses (with one-tailed probability).

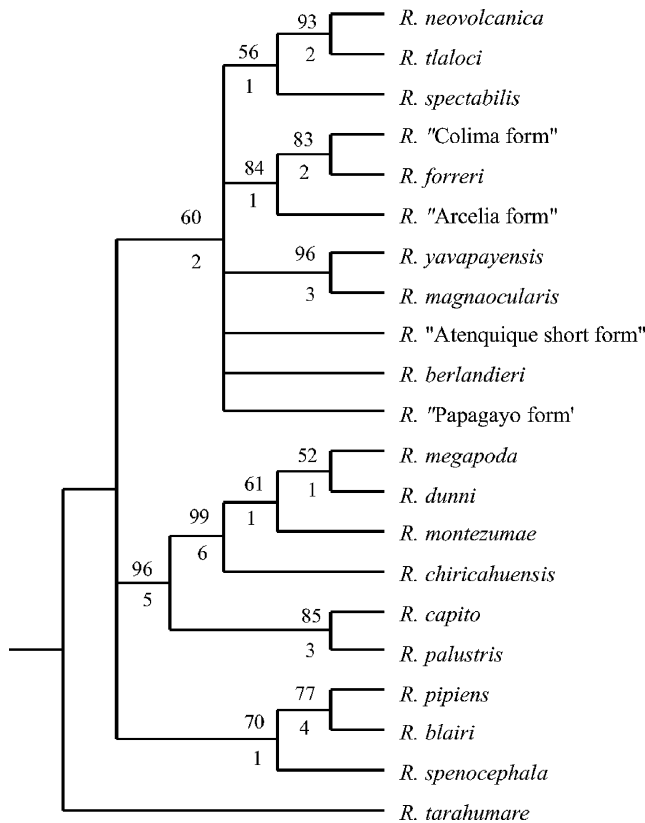


Fig. 4. Strict consensus of the 10 most parsimonious trees recovered by the analysis of the allozyme data taken from Hillis et al. (1983). Bootstrap values for the nodes retained by more than 50% of bootstrap pseudoreplicates and Bremer indices are presented above and below branches, respectively.

4. Discussion

4.1. Comparison of maximum parsimony and Bayesian analyses

In this study, the maximum parsimony and Bayesian analyses result in tree topologies with clades in common usually well supported, but conflicting relationships generally poorly supported. This coincides with findings in recent studies (Leaché and Reeder, 2002; Miller et al., 2002; Wilcox et al., 2002), that similar recovered clades with bootstrap values ≥ 80 in parsimony analyses always recovered Bayesian posterior probabilities = 1.0 in Bayesian analyses, but bootstrap values < 80 usually exhibited considerable differences compared with the Bayesian posterior probabilities. The most striking difference found between the maximum parsimony and Bayesian analyses in our study was in the common clade that comprises all the haplotypes from the Pacific region (Figs. 3a and b), which is supported by low bootstrap and Bremer support values (46 and 1, respectively) but has a Bayesian posterior probability = 1.0. In some cases, nonparametric bootstrap may underestimate the probability of a recovered clade (Hillis and Bull, 1993).

Additionally, using computer simulations Wilcox et al. (2002) found that Bayesian posterior probability values provide much more accurate estimates of clade support in comparison with nonparametric bootstrap values. In any case, whether the species from the Pacific region form a monophyletic group needs to be further investigated.

4.2. Phylogenetic relationships

Relationships derived from our mtDNA data that are concordant in the maximum parsimony and Bayesian phylogenetic hypotheses give more strongly supported clades than those obtained using Hillis et al.'s (1983) allozymic data. Strongly supported relationships derived from the maximum parsimony and Bayesian analyses do not support the monophyly of the coastal clade or the other two subgroups found by Hillis et al. (1983) for the *R. berlandieri* group. In the phylogeny obtained by these authors, a clade containing *R. berlandieri*, *R. brownorum*, and *R. sp. Papagayo* appeared as the sister group of a clade with *R. sp. Arcelia*, *R. forreri*, and *R. sp. Colima* (Fig. 1). In contrast, in our phylogenetic analysis *R. berlandieri*, *R. sp. Papagayo*, and *R. brownorum* appear in different clades, the former two being strongly supported as the sister species of *R. neovolcanica* and *R. magnaocularis*, respectively. The latter two species were nested in Hillis et al.'s (1983) phylogeny in their "Mexican Plateau" and "Foothills of the Sierra Madre Occidental" subgroupings. Moreover, our findings also differ from the results of the nuclear ribosomal DNA restriction analysis of Hillis and Davis (1986). In the phylogenetic hypothesis obtained by these authors, the included members of the *R. berlandieri* group, *R. berlandieri*, *R. spectabilis*, and *R. magnaocularis*, did not form a monophyletic group, being the latter species contained in a clade with species of the other Hillis et al.'s (1983) species groups of the *R. pipiens* complex.

4.3. Genetic and geographic variation

Based on the congruence of their strongly supported relationships, geographic provenance, and genetic divergence, the examined haplotypes from the coastal regions and contiguous lowlands and the highlands of Chiapas form eight distinct clusters (haplotypes groups 1–5, 9–10, and 13 in Fig. 3b; Table 3). There is a deep genetic divergence between haplotypes of *R. berlandieri* and *R. brownorum* (3.3–3.9%), which also exhibited different relationships. Thus, although these taxa were originally proposed to be subspecies of the same species (Sanders, 1973), they appear not to be closely related. In contrast, *R. berlandieri* and *R. neovolcanica* appear to be sister taxa and to have a shallow genetic divergence (0.8%) despite coming from widely separated geographic regions. With respect to the deepest split in the *R.*

Table 3
Ranges of genetic distances (%), using uncorrected sequence divergences; above diagonal, in parentheses) within and among the haplotypes groups proposed in this study (see Fig. 3b)

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0.8% (6)												
2	(16–21)	2.0–2.6% (1–4)											
3	(23–25)	(22–25)	2.9–3.0% (1–4)										
4	(14)	(17–18)	–	2.1–2.7% (3–4)									
5	(26–36)	(31–42)	(25)	–	3.3–4.5% (15)								
6	(43–45)	(45–46)	(51)	(38)	(43–49)	5.4–5.7% (38)							
7	(50–52)	(48–51)	(60)	(52)	(57–61)	(36)	6.3–6.5% (51)						
8	(41–45)	(42–43)	(54)	(40)	(45–53)	(36)	(51)	5.2–5.7% (43–47)					
9	(40–47)	(42–48)	(52–55)	(42–47)	(39–47)	(36–40)	(51–56)	(43–47)	5.0–5.9% (1.1% (9))				
10	(40–47)	(44–50)	(54–59)	(42–47)	(43–53)	(38–43)	(52–57)	(49–52)	5.3–6.0% (1.8–2.8% (1.1% (9)))				
11	(45–47)	(44–47)	(54)	(45)	(43–50)	(45)	(58)	(45)	6.5–6.9% (30–35)				
12	(37–39)	(37–38)	(51)	(38)	(40–49)	(39)	(54)	(43)	4.9–5.0% (32–35)	5.0–5.9% (36)			
13	(31–33)	(31–32)	(45)	(32)	(35–43)	(33)	(48)	(47)	7.1–7.4% (26–31)	6.5–7.2% (28–31)	5.7–5.9% (30)	4.7–4.9% (6)	–

brownorum clade, genetic divergence is higher among haplotypes of the two subclades (1.8–2.8%) than between haplotypes within each subclade (0.1–1.1% and 1.1%, respectively).

Relationships and genetic divergence among haplotypes from the different localities along the Pacific region show an evident geographic pattern. Haplotypes from the Sierra Madre Occidental and Sierra Madre del Sur mountain ranges (*R. sp.* “Papagayo,” *R. neovolcanica*, and *R. sp.* 1) show a considerable genetic divergence (4.8–7.7%) with respect to the haplotypes from the Pacific coastal region (*R. forreri*, *R. sp.* Arcelia, and *R. sp.* Colima) but also among themselves (4.5–6.4%). Regarding the five distinct clusters from the Pacific coast and adjacent lowlands, three of them contain exclusively southern, central and northern Pacific coast haplotypes (haplotypes groups 1, 2, and 5 in Fig. 3b, respectively), whereas the remaining two (haplotypes groups 3 and 4 in Fig. 3b) are each represented by only one haplotype from the Balsas Depression. Genetic divergences within the five clusters range from 0.1 to 1.8%, whereas divergence between clusters ranges from 2.0 to 5.5%, which exceeds some of the interspecific divergence values between the remaining species examined in this study.

4.4. Taxonomic implications

Molecular data in systematics often provide an alternative view of diversity and can expose underlying phylogenetic structure at a scale of resolution not possible with other characters (Avise, 1994, 2000). In particular, phylogenetic analyses based on mtDNA sequence haplotypes are increasingly being used to delimit species boundaries in widespread, polytypic taxa (e.g., Ashton and de Queiroz, 2001; Rodriguez-Robles and de Jesús-Escobar, 2000; Serb et al., 2001; Sullivan et al., 1997). This kind of studies may be more informative than traditional species-level taxonomies based on morphology in cases in which species have split too rapidly to allow time for many diagnostic morphological differences to evolve (Wiens and Penkrot, 2002).

According to the tree-based species delimitation approach for mtDNA sequence data proposed by Wiens and Penkrot (2002), species can be identified by being those well-supported lineages in a haplotype phylogeny that are concordant with their geography. On the basis of this criterion and the genetic distances obtained in this study, a total of 13 evolutionary species (*sensu* Wiley, 1978) appear to comprise our ingroup (haplotypes groups 1–13, Fig. 3b). However, confirmation of the new proposed lineages will require the addition of more samples that cover their whole geographic distribution, including the populations from Central America, using other complementary molecular markers that help to give a fully resolved, strongly supported topology.

Regarding the currently described species and the undescribed species identified by Hillis et al. (1983), some inferences can be made herein. Based on our data and its type locality (Presidio, Sinaloa), the name *R. forreri* should be applied only to the populations of coastal leopard frogs that occur from southern Sonora south to not more than northern Jalisco. Furthermore, although further work is needed to know the whole distribution of the species identified by Hillis et al. (1983), *R. sp. Colima* appears to inhabit from southern Jalisco south to southern Guerrero, and probably as far as central Oaxaca. In addition, *Rana sp. Papagayo*, which was previously considered as an isolated population of *R. berlandieri* (Platz, 1991), appears more closely related to the species from the different Pacific mountain ranges than to the species from the Pacific coast and Balsas region. This is supported by the fact that, like these species, *Rana sp. Papagayo* has a relatively small body size and a broken, medially displaced dorsolateral fold (Zaldívar-Riverón, personal observation).

The geographic distribution of *R. berlandieri* has been the subject of considerable confusion, mainly because of its uncertain distinctness from *R. brownorum*. The deep sequence divergence between haplotypes of *R. berlandieri* and *R. brownorum* and their different relationships indicate they actually represent separate species. Moreover, *R. berlandieri* can be morphologically distinguished from *R. brownorum* by its broken, medially displaced dorsolateral fold (the dorsolateral fold is broken but not displaced in *R. brownorum*; Sanders, 1973). Although we examined sequences of *R. berlandieri* from only two different localities, our results and morphological examination of material deposited in the Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autónoma de México suggest that this species does not extend southward beyond the central part of the state of Veracruz. Thus, the name *R. brownorum* should be applied to the populations that occur from the Gulf Coastal Plain province (at least as north as Los Tuxtlas, Veracruz) south and east through the different Sierra Madre de Chiapas subprovinces (but see above) and the Yucatan Platform province, to likely some populations in Belize and northern Guatemala.

4.5. Biogeographic inferences

The complex physiographic constitution of the Mexican territory, expressed in its extreme altitudinal variation and diverse climate, has produced the heterogeneous scenario of one of the most diverse biotas of the world (Ferrusquía-Villafranca, 1998). The Transvolcanic Belt was formed from the late Miocene to the Pliocene, approximately between 11 and 1.8 my ago (Ferrari et al., 2000). The rising of this mountain system, together with the presence of the Sierra Madre del

Sur to the south, closed off the region known as the Balsas Depression subprovince (Ferrusquía-Villafranca, 1998). Furthermore, posterior north to south rising of the Sierra de Taxco mountain range constricted the Balsas Depression almost in the middle (Toledo, 1982). The late Miocene formation of the Transvolcanic Belt and the elevation of the sea level, up to a maximum of 300 m above current levels at this time (Ewing and López, 1991), caused a division of the Gulf Coastal Plain into a north and south sides in the central area of the state of Veracruz (Pérez-Higareda and Navarro, 1980; Rosen, 1978). These phenomena have been proposed as the cause of a vicariant event that resulted in the subsequent differentiation of some mammals, reptiles, and amphibians that inhabited this region (e.g., Mulcahy and Mendelson, 2000; Pérez-Higareda and Navarro, 1980). On the other hand, the geological record suggests that the formation of the Central Depression of Chiapas occurred during the Oligocene through the Pliocene, approximately 34–1.8 my ago, due to the uplift of the Northern Mountain ranges and the Mountain Ranges of the Sierra Madre subprovinces (Stuart, 1954).

Based on the relationships recovered among the lineages herein proposed and their geographic distribution, the geological and climatic events mentioned above probably were the vicariant events that caused the current diversity of Mexican coastal leopard frogs. Further investigation of the phylogenetic relationships among the taxa that occur along the Gulf Coastal Plain and Transvolcanic Belt provinces will allow to infer if the formation of the Transvolcanic Belt and elevation of the sea level during the late Miocene to the Pliocene separated the populations of leopard frogs from these regions. However, our results suggest that these same geological and climatic events, together with the formation of the Sierra de Taxco and the mountain ranges of the Sierra Madre de Chiapas, could have split the different populations of leopard frogs that occur along the Pacific coastal region and Balsas Depression. Additionally, the close relationship between the haplotypes assigned to *R. brownorum* from the Mountain Ranges of the Sierra Madre de Chiapas and the Central Depression of Chiapas suggest a more recent separation of these subprovinces with respect to the remaining Sierra Madre de Chiapas subprovinces. A closer similarity found in previous works (Johnson, 1989, 1990) between the amphibian fauna of these two subprovinces with respect to their bordering physiographic regions support our results.

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