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Phylogeny of the New World true frogs (*Rana*)

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Abstract

Phylogenetic relationships among the species of true frogs (*Rana*) from North, South, and Central America were investigated based on the sequences of approximately 2 kb from the mitochondrial genome, sampled from most of the described species, as well as eight undescribed species. This analysis, combined with previous studies of the phylogeny of New World *Rana*, served as the basis for a revised classification of the group. The American species of *Rana* are not monophyletic; the western North American *Amerana* is more closely related to the *R. temporaria* group of Eurasia (together, these frogs form the group *Laurasiarana*). The remaining species from the Americas form the monophyletic group *Novirana*, which includes: *R. sylvatica*; *Aquarana* (the *R. catesbeiana* group); *Ranula* (the *R. palmipes* group, including the mostly upland *Levirana* species and the mostly lowland *Lithobates* species); *Torrentirana* (the *R. tarahumarae* group, or *Zweifelia*, plus *R. sierramadrensis*), *Stertirana* (the *R. montezumae* group, or *Lacusirana*, plus *R. pipiens*), *Nenirana* (the *R. areolata* group), and *Scurrilirana* (most of the southern and tropical leopard frogs). The mitochondrial sequences supported many of the previous hypotheses of relationships of New World *Rana*, although there were some differences involving the placement of the species *R. pipiens*, *R. sierramadrensis*, and *R. sylvatica*. Parametric bootstrap analyses indicated significant support for the relationships inferred from the mtDNA sequences, and rejected the previous hypotheses of relationships for these three species.
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Keywords: Phylogeny; *Rana*; Frogs; Classification; Evolution

1. Introduction

The approximately 250 extant species of true frogs (*Rana*) are found throughout much of the world, with the major exceptions being the polar regions, most of Australia, and the temperate regions of South America. About one-quarter of the species of *Rana* are found in the Americas, with the largest concentration in the southern United States and Mexico. In the New World, species of *Rana* are found from Alaska and Canada south throughout the continental United States and all of Middle America to northwestern Peru on the west side of the Andes and to eastern Brazil and northern Bolivia on the east side of the Andes. Collectively, these species are found in almost all of the major biotic provinces that are inhabited by frogs—tundra, temperate coniferous and deciduous forests, grasslands, deserts, brackish-water marshes, freshwater streams and lakes, semitropical cloud forests, and tropical rain forests.

Because one or another species of *Rana* is common throughout much of the world, several species of *Rana* have served as research subjects for a broad array of studies in evolution, ecology, behavior, development, genetics, and physiology. Given the large amount of comparative biological information available among species of *Rana*, this group has great potential for placing a wide range of biological studies in an evolutionary framework, as long as phylogenetic estimates for the group are available. This study examines the phylogeny and diversification of *Rana* in the New World based on mitochondrial DNA, and tests the significance of differences between current and previous estimates of New World *Rana* phylogeny. Our goal is to provide a

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comprehensive and well-supported phylogeny for the group to facilitate comparative studies on the biology of these frogs.

At least three species of New World *Rana* are thought to have become extinct in historic times, and several of the remaining species have undergone serious declines and are threatened with extinction. Many of the threatened species occur in the western United States and Mexico. In addition, several New World tropical species have not yet been described. This study includes all of the undescribed species of which we are aware. A second goal of this study is to provide the phylogenetic background for a revised classification of the New World *Rana*. The phylogeny will also be useful for determining priorities for conservation of the biological diversity of New World *Rana*.

The New World species are thought to form a monophyletic group together with the *Rana temporaria* species group of Eurasia (Case, 1978a; Farris et al., 1983; Hillis and Davis, 1986; Post and Uzzell, 1981; Wallace et al., 1973), and perhaps other Eurasian species groups as well (Dubois, 1992). These previous studies have supported the placement of the root of the New World *Rana* tree between the *Rana boylii* plus *Rana temporaria* groups in one clade, and the remainder of the New World *Rana* in the sister clade. Therefore, we used the *R. boylii* and *R. temporaria* groups as our outgroups to the remaining species. We included one species in the *R. temporaria* group in our study, but have otherwise restricted our analysis to the New World species (including most of the species in the *R. boylii* group). Previous studies of the phylogeny of New World *Rana* (Case, 1978a; Hillis and Davis, 1986; Hillis et al., 1983; Wallace et al., 1973) have not been comprehensive in the taxa sampled, and the phylogenetic estimates have been based on relatively small data sets. In this study, we attempted to include all extant species of New World *Rana* (described species, as well as new, but as yet undescribed, species) and examined approximately 2 kb of DNA sequences from three mitochondrial genes (large and small subunits of the ribosomal RNA genes, and the valine tRNA gene). A few described extant species were not available to us for study, but these few species are each thought to be closely related to other species that we did include in our analyses.

2. Materials and methods

2.1. Taxon sampling

We collected sequence data from 58 species of *Rana*, including all the extant described species from the New World except five species of leopard frogs that are closely related to other included species, and two members of the *Rana boylii* group that are variously treated as species or subspecies. One of the leopard frogs we failed to include was *R. megapoda*; the sample we collected to represent this species actually represents a related but undescribed species. Hillis et al. (1983) originally included this specimen (as *R. megapoda*) in a study of allozyme variation of the *R. pipiens* complex, but Webb (1996) questioned this identification and instead referred the specimen to *R. montezumae*. However, our data (including allozyme data presented by Hillis et al., 1983, and the sequence data presented in this paper) indicate that this specimen is not *R. montezumae*, but instead represents a distinct, undescribed species that is more closely related to *R. chiricahuensis* than it is to *R. montezumae*. A second leopard frog species we did not include is *R. miadis*, a species of questionable status known only from Little Corn Island, Nicaragua, and which is closely related to (and may be conspecific with) *R. taylori*. Another species missing from our analysis, *R. brownorum*, has been considered a subspecies of *R. berlandieri*, but recent evidence suggests that it should be recognized as a distinct species (Zaldívar-Riverón et al., 2004). Two additional species of leopard frogs have been described recently: *R. lemosespinali* (Smith and Chiszar, 2003), a species closely related to, and until recently included within, *R. chirichahuensis*; and *R. chichicuahutla* (Cuellar et al., 1996), a species known from a single crater lake, and which was thought by Cuellar et al. (1996) to be closely related to *R. spectabilis*. There are also three recently extinct species (*R. fisheri*, *R. johni*, and *R. pueblae*) that were unavailable for analysis (although some of the northern populations currently referred to *R. chiricahuensis* may actually be *R. fisheri*; see discussion in Section 4.6). Within the *R. boylii* group, *R. pretiosa* and *R. luteiventris* are now treated as distinct species (Green et al., 1997), although traditionally they have been considered subspecies; our geographic sampling of this complex included only populations now referred to *R. luteiventris*. Also, *R. a. aurora* and *R. a. draytonii* have been considered subspecies, but a recent analysis (Shaffer et al., 2004) suggests that they should be recognized as distinct species (we only sampled *R. a. aurora*). We also included samples of eight undescribed species, whose descriptions are forthcoming, as well as additional specimens for two species whose sequences were previously deposited in GenBank, to confirm the identity of these sequences. Taxon names, collecting localities, voucher numbers, and GenBank accession numbers are given in Table 1.

2.2. Data collection

DNA was extracted from tissues (liver, muscle, and blood) using standard phenol:chloroform extraction (Hillis et al., 1996a) or the DNEasy kit (Qiagen). Extracted DNA was resuspended in ddH_2O or in 0.1 mM Tris (pH 8.0) and quantified via gel electrophoresis and

Table 1
Specimens examined, collecting localities, voucher numbers, and GenBank accession numbers

Species	Voucher number and specimen locality data	GenBank No.
Rana areolata	KU 204370: USA: Kansas: Lyon: just S of Hartsford	AY779229
Rana aurora	MVZ 188960: USA: California: Del Norte: Kings Valley Rd., 2.4 mi N Hwy. 199	AY779196
Rana berlandieri	JSF 1136: USA: TX: Hays: San Marcos	AY779235
Rana blairi	JSF 830: USA: Kansas: Douglas: Lawrence	AY779237
Rana boylii	MVZ 148929: USA: California: Lake: along Butts Creek, 0.4 mi NW Napa County line	AY779192
Rana bwana	QCAZ 13964: Ecuador: Prov. Loja: Río Alamor near Zapotillo	AY779212
Rana capito	TNHC 60195: USA: Florida: Marion: Archibold Biological Station	AY779231
Rana cascadae	MVZ 148946: USA: California: Shasta: Dersch Meadows	AY779197
Rana catesbeiana	GenBank sequence; locality unknown	GB X12841
Rana catesbeiana	DMH 84-R2: USA: Kansas: Douglas: Lawrence	AY779206
Rana chiricahuensis	KU 194442: Mexico: Durango: Río Chico at Mexico Hwy. 40	AY779225
Rana chiricahuensis	KU 194419: USA: Arizona: Apache: Apache National Forest: Three Forks	AY779226
Rana clamitans	JSF 1118: USA: Missouri: Montgomery: 3 km W Danville	AY779204
Rana dunni	KU 194527: Mexico: Michoacan: Tintzuntzan, Lago Patzcuaro	AY779222
Rana forreri	KU 194581: Mexico: Sinaloa: 37.9 km S Escuinapa	AY779233
Rana grylio	MVZ 175945: USA: Florida: Leon: Tall Timbers Research Station, Lake Iamonia	AY779201
Rana heckscheri	MVZ 164908: USA: Florida: Gadsen-Leon: Overflow creek of Ochlocknee River at Hwy. S-12	AY779205
Rana juliani	TNHC 60324: Belize: Cayo District: Little Vaqueros Creek	AY779215
Rana luteiventris	MVZ 225749: USA: Washington: Pend Oreille: Colville Natl. Forest; Flowery Trail Road, 6.1 mi E 49 Degrees Ski area	AY779193
Rana luteiventris	MVZ 191016: USA: Montana: Lincoln: Dry Creek at Hwy. 56	AY779194
Rana macroglossa	KU 195138: Mexico: Chiapas: 7.7 km SE San Cristobal de las Casas	AY779242
Rana macroglossa	UTA A-17185: Guatemala: Sololá: Panajachel, Lake Atitlan	AY779243
Rana maculata	KU 195258: Mexico: Oaxaca: Colonia Rodulfo Figueroa, 19 km NW Rizo de Oro	AY779207
Rana magnaocularis	KU 194592: Mexico: Sonora: Arroyo Hondo, 15.2 km N Nuri	AY779239
Rana montezumae	KU 195251: Mexico: Morelos: Lagunas Zempoala	AY779223
Rana muscosa	MVZ 149006: USA: California: Mono: Meadows below Levitt Lake, W side Sonora Pass	AY779195
Rana neovolcanica	KU 194536: Mexico: Michoacan: Zurumbueno	AY779236
Rana okaloosae	toe clip (released): USA:Florida: Santa Rosa: 5 km E Harold, Garnier Creek (collected by Paul Moler)	AY779203
Rana omiltemana	KU 195179: Mexico: Guerrero: Agua de Obispo	AY779238
Rana onca	LVT 3542: USA: Nevada: Clark: Blue Point Spring, Lake Mead	AY779249
Rana palmipes	AMNH A-118801: Venezuela: Prov. Amazonas: Neblina Base Camp on Río Mawarinuma	AY779210
Rana palmipes	KU 202896: Ecuador: Prov. Napo: Misahuallí	AY779211
Rana palustris	KU 204425: USA: Indiana: Washington: Cave Creek near Campbellsburg	AY779228
Rana pipiens	GenBank sequence; locality unknown	GBX12841
Rana pipiens	JSF 1119: USA: Ohio: Ottawa: Little Portage State Park	AY779221
Rana psilonota	KU 195119: Mexico: Jalisco: 2.4 km NW Tapalpa	AY779217
Rana pustulosa	KU 200776: Mexico: Sinaloa: 2.1 km NE Santa Lucia	AY779220
Rana septentrionalis	TNHC tissue collection: Canada: Ontario: Grey	AY779200
Rana sevosa	TNHC 60194: USA: Mississippi: Harrison	AY779230
Rana sierramadrensis	KU 195181: Mexico: Guerrero: Agua de Obispo, 24.2 mi S Chilpancingo	AY779216
Rana spectabilis	KU 195186: Mexico: Hidalgo: La Estanzuela (holotype)	AY779232
Rana sphenocephela	JSF 845: USA: Kansas: Cherokee	AY779251
Rana s. utricularia	USC 7448: USA: Florida: Loop Road, Big Cypress National Preserve	AY779252
Rana subaquavocalis	James Platz Collection (specimens destroyed, DNA in TNHC tissue collection): USA: Arizona: Cochise: Ramsey Canyon	AY779227
Rana sylvatica	MVZ 137426: USA: New York: Tompkins; Connecticut Hill, ca. 10 mi SW Ithaca	AY779198
Rana sylvatica	DMH 84-R43: USA: Missouri: St. Louis: Tyson Environmental Study Area	AY779199
Rana tarahumarae	KU 194596: Mexico: Sonora: 14.4 km E Yecora	AY779218
Rana taylori	TCWC 55963: Nicaragua: Zelaya: 2.5 mi NW Rama	AY779244
Rana temporaria	DMH 84-R1: Switzerland: Valais Canton: 1.8 km NNE Grand St. Bernard Pass	AY779191
Rana tlaloci	KU 194436: Mexico: Distrito Federal: Xochimilco (paratype)	AY779234
Rana vaillanti	KU 195299: Mexico: Oaxaca: 5.6 mi NE Tapanatepec	AY779214
Rana vibicaria	MVZ 149033: Costa Rica: Prov. San José: El Empalme	AY779208
Rana virgatipes	MVZ 175944: USA: Louisiana: De Soto Parish; Frierson	AY779202
Rana warszewitschii	JSF 1127: Panama	AY779209
Rana yavapaiensis	KU 194423: USA: Arizona: Greenlee: Apache National Forest at Juan Miller Crossing	AY779240
Rana zweifeli	KU 195310: Mexico: Oaxaca: 1.6 mi S Cuyotepej	AY779219
Rana species 1	QCAZ 13219: Ecuador: Prov. Esmeraldas: 5 km W Durango	AY779213
Rana species 2	KU 204420: Mexico: San Luis Potosí: Rodeo	AY779224
Rana species 3	KU 194559: Mexico: Michoacan: 11.4 km E junction Mexico Hwy. 51 and 15	AY779250

(*continued on next page*)

Table 1 (*continued*)

Species	Voucher number and specimen locality data	GenBank No.
Rana species 4	AMNH A-124167: Panama: Chiriquí: 9 km SSE El Volcán	AY779245
Rana species 5	LACM 146764: Costa Rica: Heredia: Monte de la Cruz	AY779246
Rana species 6	LACM 146810: Costa Rica: Puntarenas: near mouth of Rio Barranca, 10 km E Puntarenas	AY779247
Rana species 7	KU 194492: Mexico: Jalisco: Contla	AY779241
Rana species 8	KU 195346: Mexico: Puebla: Río Atoyac at Mexico Hwy. 190	AY779248

AMNH, American Museum of Natural History; DMH, David M. Hillis tissue collection, University of Texas, Austin; GB, GenBank sequence; JSF, John F. Frost tissue collection, University of Texas, Austin; KU, Museum of Natural History, University of Kansas; LACM, Los Angeles County Museum; LVT, University of Nevada-Las Vegas tissue collection; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley; TCWC, Texas Cooperative Wildlife Collection, Texas A&M University; TNHC, Texas Natural History Collection, University of Texas; QCAZ, Museo de Zoología de la Pontificia Universidad Católica del Ecuador; USC, University of Southern California collection, now housed at Los Angeles County Museum; UTA, University of Texas at Arlington.

Table 2
Primers used to amplify and sequence the mtDNA 12S–16S region in this study (see also Goebel et al., 1999)

Primer name	Primer sequence (5′ → 3′)
12L1	AAAAAGCTTCAAACTGGGATTAGATACCCCA CTAT
12Sm	GGCAAGTCGTAACATGGTAAG
16Sc[a]	GTRGGCCTAAAAGCAGCCAC
16Sh	GCTAGACCATKATGCAAAAGGTA
16Sa[a]	ATGTTTTTGGTAAACAGGCG
16H1	CTCCGGTCTGAACTCAGATCACGTAGG

Primers 12L1, 16Sh, 12Sm, and 16H1 were used in primary amplifications. Other primers were used for sequencing

[a] Reverse primers also used in sequencing.

comparison to a known standard. Approximately, 2 kb of mitochondrial DNA, spanning the region from 12S rRNA through 16S rRNA, including the intervening valine-tRNA (positions 442–2400 of the *Rana pipiens* mtDNA, GenBank Y10945), were PCR amplified using a series of nested primers (Table 2). PCR products were gel-purified and directly sequenced using fluorescent thermal-cycle sequencing and an ABI 377 automated sequencer (Perkin–Elmer).

2.3. Phylogenetic analysis

DNA sequences, ranging in length from 1935 to 1962 bp, were obtained from 64 individuals representing 58 species. Each sequence was examined for the presence of conserved secondary structural elements to ensure the sequences were valid, coding mtDNA sequences. Sequences were aligned using Clustal W (Thompson et al., 1994) and manually adjusted to accommodate secondary structural elements (Cannone et al., 2002; http://www.rna.icmb.utexas.edu/). Positional homology was uncertain for 38 bp, and these positions were excluded from the final analysis (http://www.treebase.org/; SN2065-7080).

Aligned sequences were analyzed using PAUP* (v4.0b4-b8, Swofford, 2000) under the maximum-likelihood (ML) and parsimony (MP) criteria, and using a parallel version of a genetic algorithm for maximum likelihood (Brauer et al., 2002). For ML analyses, the GTR + Γ + PINVAR model of sequence evolution, with four gamma-distributed rate categories, was chosen for the analysis (Appendix A). This was the preferred model for the data found using the stepwise likelihood-ratio test procedure described by Posada and Crandall (2001). Starting trees for maximum likelihood searches in PAUP* were obtained by conducting 5000 parsimony searches from different stepwise-addition trees (using random taxon addition), followed by TBR branch swapping. From these 5000 searches, 10 of the best trees under the parsimony criterion were selected as starting trees for the maximum likelihood searches, and TBR branch swapping (to completion) was used to search for optimal trees under the likelihood criterion. We also conducted eight independent analyses using the parallel genetic algorithm (Brauer et al., 2002), starting from randomly selected trees, under the GTR + Γ + PINVAR model. The best likelihood solution that we found with each searching method (branch swapping in PAUP* from 10 parsimony starting trees, and selection under the genetic algorithm from eight samples of random trees) was identical. Bayesian posterior probabilities (bpp) were estimated for each branch of our best likelihood tree using MrBayes (v3.0b4, Huelsenbeck and Ronquist, 2001), also using the GTR + Γ + PINVAR model. Four analyses, with Monte Carlo Markov chain (MCMC) length of five million generations each, were conducted. The log-likelihood scores were found to consistently stabilize after 500,000 generations within and among these four independent analyses. Therefore, the initial 500,000 generations from each run were discarded, and we sampled one out of every 50 generations from the remaining 18 million generations (across all four independent analyses) to calculate posterior probabilities for each branch in the maximum likelihood tree. If a bipartition's posterior probability was $\geqslant 95\%$, it was considered significantly supported. The interpretation of Bayesian posterior probabilities for branches in a phylogenetic analysis is well-defined; they represent the probability that the corresponding clade is present in the true tree, given the data examined, the likelihood model, and the specified priors (Huelsenbeck and Rannala, 2004; Larget and Simon, 1999). In contrast, there is no clear or widely accepted interpretation of other commonly used

measures of phylogenetic support, such as nonparametric bootstrap proportions or decay indices, and their use in the context of statistical testing is not straightforward (e.g., see Hillis and Bull, 1993; Swofford et al., 1996; Newton, 1996; Wilcox et al., 2002; and Huelsenbeck and Rannala, 2004). Therefore, we prefer to use Bayesian posterior probabilities over other measures of phylogenetic support that have no widely accepted statistical interpretation.

2.4. Testing alternative hypotheses

Wherever our phylogenetic findings differed from existing hypotheses about the relationships of New World *Rana*, we conducted parametric bootstrap tests on the results to determine if the differences between the competing hypotheses were significantly different (Goldman et al., 2000; Hillis et al., 1996b; Huelsenbeck et al., 1996). We prefer parametric bootstrap tests over other (nonparametric) approaches for testing a priori hypotheses in phylogenetic analysis because of the greater power of the parametric tests (Goldman et al., 2000; Huelsenbeck et al., 1996). In each case, we constrained our analysis to fit the hypothesis that was to be tested, and found the best tree that supported the hypothesis in question. We then estimated parameters for a model of evolution from the observed data, and used these parameters and the optimized tree topology to simulate 100 replicates of the model tree (using Seq-Gen version 1.2.5; Rambaut and Grassly, 1997). We then analyzed each replicate and compared the best-fit tree to the best tree that fit the constraint of the null hypothesis. This procedure allowed us to construct expected distributions of the test statistic (in this case, the difference in tree length under the parsimony criterion) under the assumption that the null hypothesis was true. If the observed difference in tree length (between our observed best-fit tree and the tree that best fit the hypothesis under test) was greater than 95% of the simulated values, then the null hypothesis was rejected.

3. Results

3.1. Phylogenetic analysis

The best-fit tree based on the maximum likelihood analysis is shown in Fig. 1, with all branches that have significant support (posterior probability >95%) indicated by asterisks. This tree has a log-likelihood score of −21812.64132. All of the PAUP* and genetic algorithm searches found this tree or another tree of similar but slightly lower score (−21812.66941); this latter tree differed only in rearrangements of some of the weakly supported clades within the tropical leopard frogs (*Scurrilirana*). The Bayesian analysis indicated significant support for 47 out of 61 internal branches. All but one of the 14 branches that lacked significant support united species within recognized species groups: inside the *R. boylii* group (one branch), within the *R. catesbeiana* group (three branches), and within the *R. pipiens* group (nine branches). The relationships among the recognized species groups were all significantly supported, with the single exception of the sister-group relationship of the *R. tarahumarae* and *R. pipiens* species groups (Fig. 1).

We found four shortest parsimony trees of length 4121; these four trees were far from optimal under the likelihood criterion (log likelihood scores for these four trees ranged from −21886.27 to −21903.92, or about 74–91 log likelihood units from the maximum likelihood estimate). These four trees differ from the maximum likelihood tree shown in Fig. 1 in the following ways: (1) they join the relatively long branches that lead to *R. temporaria* and *R. boylii* together, and so do not support the monophyly of the *R. boylii* group; (2) they join the relatively long branches that lead to *R. grylio* and *R. virgatipes* together (within the *R. catesbeiana* group); (3) they group the Venezuelan sample of *R. palmipes* (another long-branch taxon) outside of the *R. palmipes* group, as sister to the *R. pipiens* group; (4) they show the remaining members of the *R. palmipes* group as paraphyletic with respect to the *R. tarahumarae* plus *R. pipiens* groups; and (5) they show minor rearrangements involving some of the weakly supported branches within the *R. pipiens* group. With the exception of the rearrangements within the *R. pipiens* group (which reflect weak support under either criterion), all of the other differences between the maximum likelihood and maximum parsimony trees are consistent with long-branch attraction problems in the parsimony tree (Felsenstein, 1978; Huelsenbeck and Hillis, 1993). Because the parsimony trees show significantly worse fit to the data compared to the maximum likelihood tree when the details of the model of sequence evolution are taken into account, we consider the maximum likelihood solution shown in Fig. 1 to be our best estimate of phylogeny for the group.

Given the general correspondence of morphology, allozymes, immunology, and DNA sequences for many of the major clades of New World *Rana* (Case, 1978a; Hillis, 1987, 1988; Hillis and Davis, 1986; Hillis and de Sá, 1988; Hillis et al., 1983, 1984; this study), we have presented a phylogenetic classification of this group in Appendix B (summarized in Fig. 2). This classification preserves and defines previously named groups within *Rana*, wherever these names are applicable. In some cases, there have not been any formal names applied to some well-supported groups, and in these cases we have provided new clade names. Following the principles of phylogenetic classification (de Queiroz and Gauthier, 1990, 1992, 1994), the clade names presented in Appendix B are all unranked (i.e., they are not assigned to categories such as section or subgenus). However, their

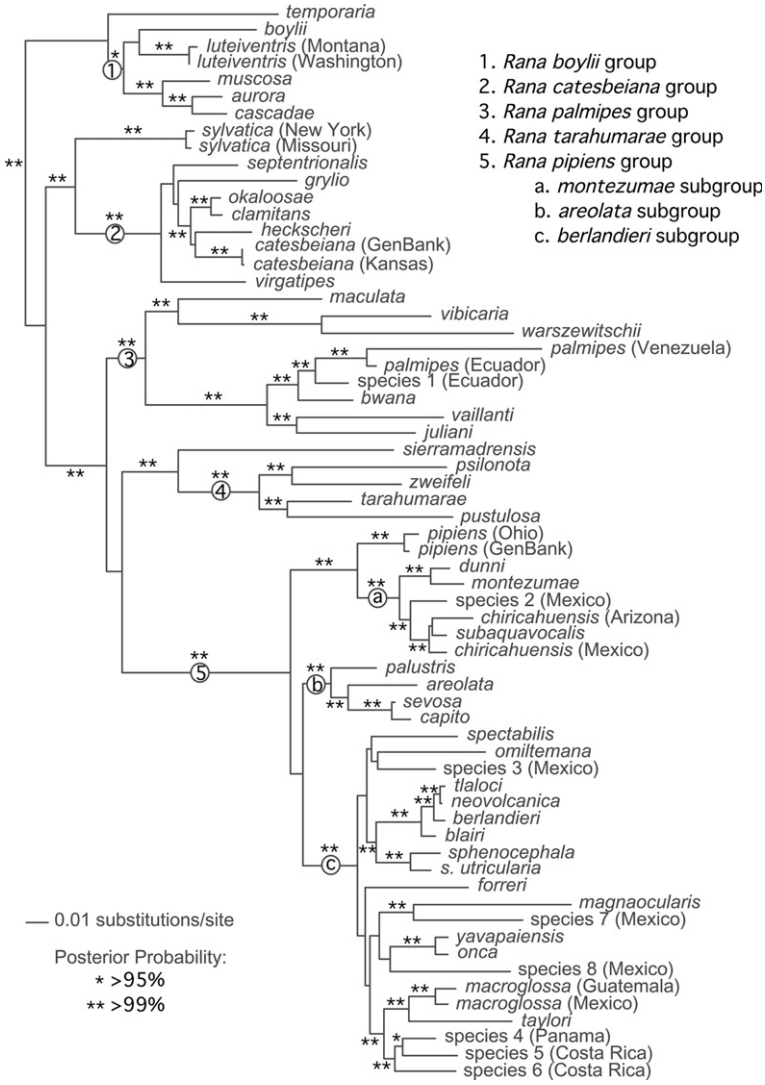

Fig. 1. Phylogenetic tree of New World *Rana* based on maximum likelihood analysis of rDNA sequences. The scale bar indicates divergence. The numbers and letters on clades represent some of the traditionally recognized groups discussed in the text. Three species (*R. pipiens*, *R. sierramadrensis*, and *R. sylvatica*) do not group with their traditional species groups in our analysis; the placement of these three species was subjected to additional testing. Branches with two asterisks were supported by posterior probabilities of >99%; branches with one asterisk were supported by posterior probabilities of >95%.

hierarchical relationships are indicated by indenting and also can be seen in Fig. 2. Nonetheless, under the rules of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature, 1999), any uninominal name of a "… genus-group division of a genus, even if it is proposed for a secondary (or further) subdivision, is deemed to be a subgeneric name even if the division is denoted by a term such as 'section' or 'division'…" (Art. 10.4). Therefore, all of the clade names within *Rana* that are defined in Appendix B are subgenera under ICZN rules, even though the clades are nested hierarchically within one another (Hillis et al., 2001). We recommend that *Rana* still be the primary clade name used with species epithets to promote nomenclatural stability; the other clade names, in turn, are useful for discussing historical groups of species within *Rana*. Therefore, the species names in this paper (together with the clade name *Rana*) are identical under either traditional Linnean binomial nomenclature (as binomials), or following Option M for phylogenetic species names as suggested by Cantino et al. (1999).

The tree in Fig. 1 supports many of the traditional species groups that have been recognized previously, including the *R. boylii* group (named *Amerana* by Dubois, 1992; see Appendix B for the definition of this and other clade names used in this paper), the *R. catesbeiana* group (*Aquarana*), the *R. tarahumare* group (*Zweifelia*), the *R. palmipes* group (*Ranula*; but see below for discussion of the relationships of *R. sierramadrensis*), the *R. pipiens* complex (*Pantherana*), the *R. montezumae* group (*Lacusirana*), and the *R. areolata* group (*Nenirana*). The *R. pipiens* complex (now *Pantherana*) was

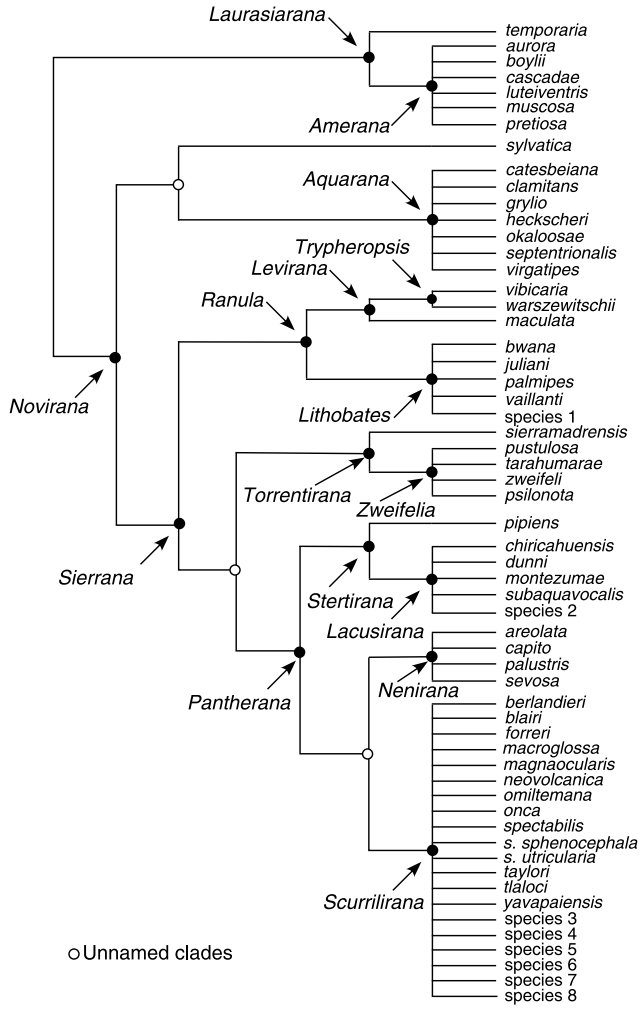

Fig. 2. Classification of New World *Rana*. Names are defined and discussed in Appendix B.

divided into the α and β divisions by Hillis et al. (1983) on the basis of allozyme variation. The α division contained *Lacusirana* and *Nenirana*, whereas the β division contained *Scurrilirana* and *R. pipiens*. In our analysis of DNA sequences, the major exception to this proposal is that *R. pipiens* appears to be the sister species of *Lacusirana*, rather than closely related to (or even a member of) *Scurrilirana* (Fig. 1). Without the inclusion of *R. pipiens*, the support for *Scurrilirana* is strong (100% Bayesian posterior probability). However, even a modified alpha division that includes *R. pipiens* (*Nenirana* plus *Stertirana*) appears paraphyletic on the optimal tree, with *Nenirana* more closely related to *Scurrilirana* than to *Stertirana* (although this latter relationship is not significantly supported).

3.2. Tests of previous hypotheses

Although our phylogenetic hypothesis and classification shown in Fig. 1 is broadly consistent with recent summaries of the phylogeny of New World *Rana* based on other evidence (Hillis, 1988; Hillis and Davis, 1986; Hillis and de Sá, 1988; Hillis et al., 1983, 1984), there are three significant differences. These differences do not depend on the optimality criterion selected, as they appear in both the maximum likelihood and maximum parsimony trees. The phylogenetic analysis of mitochondrial DNA supports each of the following:

1. *Rana sylvatica* as the sister-group of *Aquarana*, rather than as the sister-group (or a part of) *Laurasiarana* (contra Farris et al., 1980, 1983, based on analysis of immunological data; and also contra Hillis and Davis, 1986, based on analysis of nuclear ribosomal DNA restriction sites).
2. *Rana pipiens* as the sister-group of *Lacusirana*, rather than as a part of *Scurrilirana* (contra Hillis et al., 1983, based on analysis of allozyme data).
3. *Rana sierramadrensis* as the sister-group of *Zweifelia*, rather than as a part of *Ranula* (contra Hillis and de Sá, 1988, based on morphological analysis).

Our parametric bootstrap tests of these three a priori hypotheses (Fig. 3) showed that each could be rejected at $p < 0.05$ based on the mitochondrial DNA sequences. However, the support for *R. sylvatica* as the sister-group to *Aquarana* (rather than within or sister to *Laurasiarana*) was relatively weak, with the observed difference in tree length just greater than needed to reject the previous hypothesis ($p = 0.046$). In contrast, the previous hypotheses for the relationships of *R. pipiens* ($p < 0.002$) and *R. sierramadrensis* ($p < 0.014$) were rejected easily.

4. Discussion

4.1. Relationships of Rana sylvatica

The relationship of *Rana sylvatica* to other species of the New World *Rana* has long been controversial. In general appearance, this species resembles species of the *R. temporaria* group (of *Laurasiarana*), and many authors have long assumed that *R. sylvatica* was simply a North American member of this otherwise Eurasian species group. Likewise, *Amerana* of western North America has been considered to be closely related to, or even a part of, the *R. temporaria* group (Farris et al., 1980, 1983). However, Case (1978a) suggested that *R. sylvatica* was more closely related to the eastern and tropical groups of North American *Rana* (what is now *Novirana*) than to the western American *Amerana* or the Eurasian *R. temporaria* group, based on immunological comparisons. This conclusion was contested by Farris et al. (1980, 1983). Post and Uzzell (1981) made additional immunological comparisons, and suggested that the data best supported either a relationship to the eastern and tropical *Rana* (as suggested by Case, 1978a), or

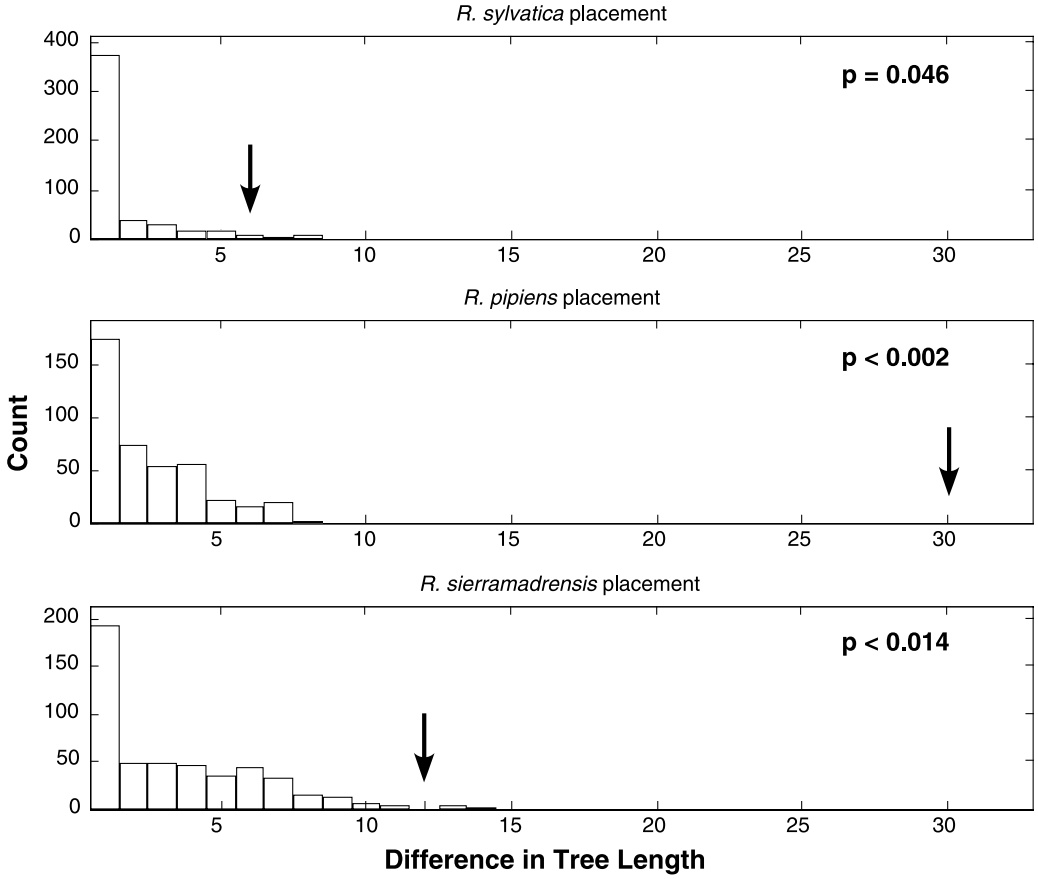

Fig. 3. Results of the parametric bootstrap tests of three hypotheses (see text). In each case, the graphs show the difference (in parsimony score) between the null and test hypothesis for each of 500 replicates (histogram), as well as the observed difference in score for the empirical data (arrow). In each case, the null hypothesis can be rejected at $p < 0.05$.

else a placement of *R. sylvatica* as a sister-group to all the other New World *Rana* plus the *R. temporaria* group. Hillis and Davis (1986) supported the placement of *R. sylvatica* with the *R. temporaria* group on the basis of nuclear ribosomal RNA restriction sites.

Our optimal tree (Fig. 1) places *Rana sylvatica* as the sister species to *Aquarana*. Although this placement is not exactly like that proposed by Case (1978a), it is closer to that proposal than any other previously suggested. The relationship of *R. sylvatica* to *Aquarana* was supported in 100% of the trees sampled in the Bayesian analysis, as well as in the most-parsimonious trees. Moreover, we could reject the alternative placement of *R. sylvatica* with *Laurasiarana* (either as a sister-group, or embedded within *Laurasiarana*) in the parametric bootstrap analysis ($p = 0.046$). However, given the apparent conflict with previous studies and other data sets, we have refrained from suggesting any changes to the classification of *Rana* that involve the placement of *R. sylvatica*.

4.2. Amerana (*The* Rana boylii *group*)

This group is well-supported by our likelihood analysis, although the relationship of *Amerana* to the *R. temporaria* group of Eurasia requires further investigation and taxon sampling (Green, 1986b). In our analysis (with only a single representative of the *R. temporaria* group, however), *Amerana* is monophyletic. Although almost all previous studies have supported the monophyly of *Amerana* (see summary presented by Macey et al., 2001), there has been less agreement on the relationships among the species within this group (Case, 1978a,b; Farris et al., 1980, 1983; Green, 1986a,b; Hillis and Davis, 1986; Macey et al., 2001; Post and Uzzell, 1981; Shaffer et al., 2004; Wallace et al., 1973; Zweifel, 1955). The close relationship between *R. pretiosa* and *R. luteiventris* has been reflected in the treatment of these two taxa as subspecies until recently. Other relationships in the group, however, are in conflict among data sets, including the relationship of the two taxa variously treated as distinct species or as subspecies of *R. aurora* (*R. aurora* and *R. draytonii*). Allozyme data presented by Case (1978b) and Green (1986b), as re-analyzed by Macey et al. (2001), support the sister-group relationship between *R. aurora* and *R. cascadae* shown in Fig. 1, but this relationship is contradicted by some karyotypic, immunological, and other allozyme analyses (Case, 1978a,b; Green, 1986a). Analysis by Macey et al. (2001)

of mitochondrial genes (different from the genes we sampled) support a tree very similar to the tree we found for the *Amerana* species. They found strong support for a monophyletic *Amerana*, weak support for a relationship between *R. boylii* and *R. luteiventris* (reported as *R. pretiosa*), and strong support for a clade consisting of *R. muscosa*, *R. aurora*, and *R. cascadae* (as on our tree). The only apparent difference between our two trees is that we found strong support for a sister-group relationship between *R. aurora* and *R. cascadae*, whereas Macey et al. (2001) reported weak support for a sister-group relationship between *R. cascadae* and *R. muscosa*. However, this difference appears to result from the different samples we used for "*R. aurora*." Our sample represents *R. a. aurora*, whereas the Macey et al. (2001) sample represented *R. a. draytonii*. Shaffer et al. (2004) found recently that *R. aurora* and *R. draytonii* are not each other's closest relatives, and should be recognized as distinct species. Moreover, Shaffer et al. (2004) found that *R. aurora* (sensu stricto) and *R. cascadae* are sister species (as also supported by our data), but could not resolve the trichotomy among this pair of species, *R. draytonii*, and *R. muscosa*. Thus, taking the different taxa sampled across each of the studies into account, our tree for *Amerana* does not appear to be in conflict with the results of the other DNA sequence studies on this group (Macey et al., 2001; and Shaffer et al., 2004). None of our studies are in conflict with the tree (((*pretiosa*, *luteiventris*) *boylii*) (((*aurora*, *cascadae*) *muscosa*) *draytonii*)), although none of the DNA studies to date include samples of all of these taxa.

Rana pretiosa (together with *R. luteiventris*) sometimes has been supported as the sister-group to the remaining species of *Amerana* (weak support from allozyme data and DNA restriction analysis of nuclear rRNA genes), although our analysis weakly supports a relationship of these taxa to *R. boylii* (Fig. 1). Macey et al. (2001) also found weak support for a relationship between *R. pretiosa*/*luteiventris* and *R. boylii*. Green (1986b) suggested some form of "mosaic evolution" to account for the discrepancies of relationships of this group supported among the various data sets. However, there does not appear to be any strong conflict between any of the data sets presented to date and the tree for *Amerana* shown in Fig. 1, once taxon sampling issues involving the *R. aurora*/*draytonii* complex are taken into account.

4.3. Aquarana (*The* Rana catesbeiana *group*)

Our data strongly support the monophyly of the *Aquarana*. This group is also supported on the basis of immunological data (Case, 1978a), morphology (Hillis, 1985), allozymes (Pytel, 1986), and nuclear rDNA restriction sites (Hillis and Davis, 1986). In addition to the monphyly of *Aquarana*, our analysis strongly supports a close relationship between *R. clamitans* and *R. okaloosae*, and a group that consists of these two species plus *R. catesbeiana* and *R. heckscheri* (Figs. 1 and 2). Each of these relationships was also supported in a more extensive analysis of this group by Austin et al. (2003). Austin et al. (2003) showed that *R. okaloosae* is phylogenetically embedded within *R. clamitans*, and suggested a very recent origin for *R. okaloosae*.

4.4. Ranula (*The* Rana palmipes *group*)

Hillis and Davis (1986) reported a single nuclear rDNA restriction site that supported the monophyly of the *Rana palmipes* group, and additional sites that supported the monophyly of the lowland members of the group and the montane members of the group, exclusive of *R. sierramadrensis*. Hillis and de Sá (1988) reviewed the *R. palmipes* group based on morphological analyses of adults and tadpoles. They divided the group into two subgroups: one containing the lowland species (*R. bwana*, *R. palmipes*, and *R. vaillanti*) and the other containing the montane/upland species (*R. juliani*, *R. maculata*, *R. sierramadrensis*, *R. vibicaria*, and *R. warszewitschii*). Our analysis supports this arrangement, with two major exceptions. First, *R. sierramadrensis* does not appear to be a member of *Ranula*. Instead, the mtDNA data strongly place it as sister to the *R. tarahumarae* group (*Zweifelia*). Second, *R. juliani* appears to be a part of the lowland clade (*Lithobates*), rather than the upland clade (*Levirana*). Two of the morphological characters that Hillis and de Sá (1988) used to place *R. juliani* with the species of *Levirana* were features of the tadpole mouthparts (the presence of larval marginal teeth, and an increase in the number of upper rows of teeth from 4 to 5–7). Both of these features are correlated with tadpoles in montane streams, and it appears that the tadpoles of *R. juliani* are convergent with the tadpoles of species *Levirana* (and divergent from the pond tadpoles of the more closely related species of *Lithobates*). If the mitochondrial DNA tree is correct, there is also convergence in two characters of coloration: the supralabial stripe and dark face mask. The convergence in morphology of *R. juliani* is strongest with *R. maculata*, and indeed the first reports of *R. juliani* (from the Maya Mountains of Belize; Henderson and Hoevers, 1975; Lee, 1976) identified specimens of this species as *R. maculata*. However, the mitochondrial DNA data reject a close relationship among the montane species, and instead suggest that *R. juliani* is a secondarily montane species otherwise related to the widespread lowland species in *Lithobates*.

A number of authors have noted that the putative widespread species *Rana palmipes* probably consists of many species (Cochran and Goin, 1970; Fowler, 1913; Günther, 1900; Hillis and de Sá, 1988). Hillis and de Sá (1988) removed *R. vaillanti* from the synonymy of *R.*

palmipes, and described *R. bwana* from southwestern Ecuador and northwestern Peru. *Ranula gollmeri* was described by Peters (1859) based on a juvenile specimen from Caracas, Venezuela, although the species was almost immediately considered a synonym of *Rana palmipes*, even by Peters (Boulenger, 1920). The name *Rana gollmeri* is available if the northern South American populations are determined to be distinct. We have not sampled sufficiently to answer this question, although our sample of "*R. palmipes*" from Venezuela is considerably more divergent from our Ecuadorian sample of *R. palmipes* than are many other recognized sister species of *Rana* in our analysis (the Venezuelan sample does not even group within *Ranula* in the parsimony analyses, although this is likely an artifact of its high degree of overall divergence). We are also aware of an additional undescribed species in *Lithobates*, distributed in northwestern Ecuador and western Colombia (species 1 in Figs. 1 and 2). This species was referred to *R. vaillanti* by Hillis and de Sá (1988), but they noted that the tadpoles of species 1 are darkly pigmented and easily distinguished from those of *R. vaillanti*. Our phylogenetic analysis suggests that this species is more closely related to *R. palmipes* than to *R. vaillanti*. Thus, the clade *Lithobates* may contain six (or more) essentially parapatric species: *R. vaillanti* throughout much of lowland Central America, north to the state of Veracruz, Mexico; *R. juliani* in the Maya Mountians of Belize (and presumably adjacent Guatemala); *R. palmipes* in the Amazon River basin; *R. bwana* along the dry Pacific coast of northwestern Peru and southwestern Ecuador; possibly *R. gollmeri* in northern South America (if this species is supported as distinct by additional analysis); and an undescribed species (species 1) along the wet Pacific coast of northwestern Ecuador and western Colombia. Interestingly, the geographic ranges of these species mirror the ranges of other widespread lowland complexes of frogs, such as the *Physalaemus pustulosus* group (Cannatella et al., 1998).

4.5. Torrentirana (*The* Rana tarahumarae *group*)

Members of the *Rana tarahumarae* group are found throughout the Sierra Madre Occidental and Sierra Madre del Sur of western Mexico, and historically north to southern Arizona in the United States. Two species (*R. johni* and *R. pueblae*) were also formerly found in the Sierra Madre Oriental in the states of San Luís Potosí, Hidalgo, and Puebla, Mexico, but neither species has been seen since the 1970s and both are believed to be extinct (Hillis et al., 1984). Populations of *R. tarahumarae* have also rapidly declined, and the species has been extirpated from its former range in southern Arizona (Webb, 2001). In addition, populations of *R. sierramadrensis* disappeared from many former localities in Oaxaca and Guerrero, Mexico by the mid-1980s, and some former populations of *R. pustulosa* in Sinaloa, Mexico disappeared by the mid-1990s (DMH, personal observation). The species called "southern *R. tarahumarae*" by Hillis et al. (1984) was described as *R. psilonota* by Webb (2001). Currently, *R. zweifeli* and *R. psilonota* may be the only species in this group with relatively widespread and healthy populations.

All members of this species group lack vocal sacs and slits, have reduced or absent external tympana, and no calls have been recorded for any of the species. However, there are also no published observations of breeding among any of the species in this group. Given that the species in this group are highly aquatic and typically occupy montane streams and plunge pools, it is likely that calls, if they are produced at all, are produced below the surface of the water.

4.6. Pantherana (*The* Rana pipiens *complex*)

The leopard frogs (*Pantherana*) have a complex systematic history (Hillis, 1988). Many of the currently recognized species were once thought to represent a single, widespread species that ranged from Canada to Panama (Moore, 1944). However, many of these species were found to occur sympatrically, with little or no hybridization (Littlejohn and Oldham, 1968; Moore, 1975; Pace, 1974). In addition, the species that were once placed into the wide ranging "*R. pipiens*" are not each others' closest relatives. The species have since been found to be morphologically, behaviorally, phylogenetically, physiologically, and ecologically distinct (Hillis, 1988). However, there are still a large number of taxonomic problems within the group. Several species (especially in Mexico and Central America) remain to be described (Fig. 1; see also Zaldívar-Riverón et al., 2004), and other currently recognized species appear to be conspecific. In particular, *R. neovolcanica* and *R. tlaloci* are extremely closely related and may be conspecific, although this may prove difficult to study because of the disjunct range of *R. tlaloci* and the fact that these isolated populations may now be extinct because of the growth of Mexico City (Hillis and Frost, 1985). Also closely related are *R. onca* and *R. yavapaiensis*, as well as *R. sevosa* and *R. capito*. Each of these pairs of species are more similar in their mtDNA sequences than are *R. s. sphenocephala* and *R. s. utricularia*, which are currently recognized as subspecies. Additional study is needed to clarify the status of these closely related taxa. In addition, the recognition of *R. subaquavocalis* as distinct from *R. chiricahuensis* is not well supported. On the other hand, the populations of leopard frogs from the Mogollon Rim in Arizona that are currently recognized as *R. chiricahuensis* are morphologically distinct from *R. chiricahuensis* in southern Arizona, New Mexico, and Mexico, and may be referable to *R. fisheri* (a species described from southern Nevada, and considered extinct by many authors). *Rana*

fisheri appears to have been closely related to the Mogollon Rim populations of "*R. chiricahuensis*" based on morphological similarity, and the name *R. fisheri* may be applicable to these Mogollon Rim leopard frogs. Our sample of *R. chiricahuensis* from Arizona is from the Mogollon Rim, and therefore perhaps should be referred to *R. fisheri*. However, we have followed the current taxonomic practice of referring to these frogs as *R. chiricahuensis*, pending detailed analysis of the problem.

Although some of the currently described species of *Pantherana* appear to be very closely related to one another (such as the *R. onca*/*R. yavapaiensis* species pair, the *R. neovolcanica*/*R. tlaloci* species pair, and the *R. sevosa*/*R. capito* species pair), there are still a number of undescribed leopard frog species in Mexico and Central America that are relatively distantly related to any described species (e.g., species 2–8 in Fig. 1). More thorough sampling and analysis is needed to determine the status and distribution of many of these taxa, however.

Hillis et al. (1983) recognized two major divisions of *Pantherana* (then the *R. pipiens* complex), which they informally termed the alpha and beta divisions. Their α division consisted of two species groups, the *R. montezumae* group (here named *Lacusirana*, Fig. 2 and Appendix B) and the *R. areolata* group (here named *Nenirana*, Fig. 2 and Appendix B). The other leopard frogs were placed in the β division, which was further divided into the *R. pipiens* group (not supported in our analysis) and the *R. berlandieri* group (essentially *Scurrilirana* of Fig. 2 and Appendix B, but now also including *R. blairi* and *R. sphenocephala*). Our results differ from those of Hillis et al. (1983) most clearly in the placement of *R. pipiens*, which Hillis et al. (1983) considered to be a member of the β division. However, the mtDNA sequence analysis strongly rejects a relationship of *R. pipiens* to *Scurrili- rana* (Fig. 3), and instead places *R. pipiens* as the sister species of *Lacusirana* (together these taxa comprise *Stertirana*).

The phylogenetic position of *R. pipiens* has bearing on the evolutionary reconstruction of advertisement call evolution in leopard frogs. The advertisement calls of many species of *Pantherana* are highly complex, and consist of many distinct elements. These elements of the advertisement calls have been described as mating trills or snores, chuckles, grunts, and grinds (e.g., Larson, 2004; Mecham, 1971; Pace, 1974; Schmidt, 1968). The "mating trill" or "mating call" element (called a snore by Larson, 2004) has been associated with mate attraction by most authors, whereas other elements have been suggested as having a territorial or aggressive function (Frost and Platz, 1983; Littlejohn and Oldham, 1968; Mecham, 1971; Pace, 1974; Schmidt, 1968). Larson (2004) emphasized that these functions have not been confirmed experimentally, although Oldham (1974) did show some female response to the "mating call" in *R. sphenocephala*. In addition, the "mating call" element of the advertisement call is the only element that is produced by all species of *Pantherana* during breeding choruses, and some species are not known to produce the other elements. Therefore, this element seems likely to function in female attraction, although it is possible that other elements may also contribute to this role in some species. All of the species of *Stertirana* (including *R. pipiens*) and *Nenirana* have a "snore-like" mating call (see Fig. 4, and Mecham, 1971; Schaaf, 1971; and Altig and Lohoefener, 1983 for examples). Larson (2004) provided an explicit definition of the snore type of call; in brief, a snore consists of a rapid, relatively uniform pulse rate, with a long series of continuous pulses (typically 30–80 in the various species) that are modulated in amplitude and frequency. In contrast, the species of *Scurrilirana* all have some form of "chuckle-like" mating call, that superficially resembles some of the other elements of the advertisement call of species such as *R. pipiens*. The "chuckle-like" calls of species of *Scurrilirana* consist of a

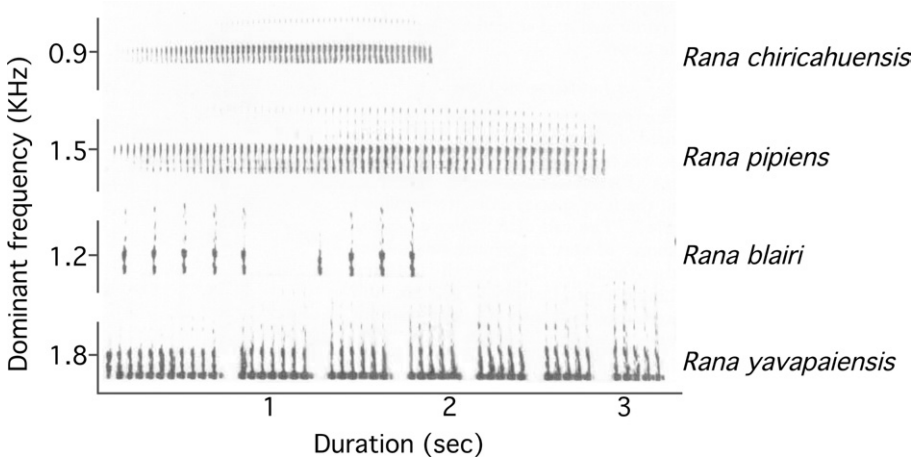

Fig. 4. Audiospectrograms of the "mating call" component of the advertisement calls of four species of *Pantherana* (adapted and redrawn from Frost and Platz, 1983). The calls of the top two species (*R. chiricahuensis* and *R. pipiens*) are examples of the snore-type call pattern, and the calls of the lower two species (*R. blairi* and *R. yavapaiensis*) are examples of the chuckle-type call pattern.

series of groups of pulses of variable number (typically 4–12), each group with a slower pulse rate compared to a snore, and greater spacing between successive groups than between successive pulses (see examples in Fig. 4). As in a snore, these pulses are often modulated in frequency and amplitude, and the calls are sometimes likened to the sound of rapid human laughter. The chuckle calls of *Scurrilirana* nonetheless vary widely among species in pulse rate, pulse number, dominant frequency, and spacing of note sequences. Based on the phylogeny shown in Fig. 1, the long, continuous snore-like mating call is ancestral for *Pantherana*, but has been modified into a chuckle-like mating call in species of *Scurrilirana*. However, *Rana pipiens* has been demonstrated to have a complex call that often consists of both a snore (identified by most authors as the mating call) as well as chuckle-like elements and grunts (Larson, 2004). In contrast, only the snore has been reported in the advertisement call of most species of *Lacusirana* and *Nenirana*. The presence of the diverse elements in the advertisement call of *R. pipiens*, combined with the phylogenetic location of this species as the sister-species to *Lacusirana* (Fig. 1), suggests the possibility that different elements of the ancestral call of *Pantherana* may have been selected to function in mate attraction in the various species of this group. The various advertisement calls of the species of *Pantherana* are among the most complex and diverse calls of any anurans, and this group has undergone rapid speciation and diversification (Fig. 1). Together, these facts indicate that this would be an ideal group for the study of call evolution as it relates to reproductive isolation.

5. Conclusions

The phylogenetic estimate shown in Fig. 1 provides an opportunity for the comparative study of many aspects of the biology of species of New World *Rana*. The species in this group are highly diverse in ecology, physiology, and behavior, and many of the species are common enough to be ideal subjects for intensive biological study. As an example, the group as a whole would serve as an excellent model system for the study of many aspects of call evolution in frogs. There is considerable variation in the types and diversity of calls that are produced among the major clades, with many species exhibiting complex calls that appear to have several different functions. Within some of the clades, there are many closely related species that have recently split, and calls appear to be playing an important role in speciation (Littlejohn and Oldham, 1968). The morphological structures that are associated with call production and detection (such as vocal sacs, vocal slits, and external tympana) are highly variable across species groups and species, and in some cases even the presence or absence of vocal sacs and slits is polymorphic within species (Hayes and Kremples, 1986; Hillis and de Sá, 1988). Species of New World *Rana* vary in other aspects of call production, including whether the calls are produced in air or under water, and how the calls function in mate attraction. Given all the diversity (both intraspecific and interspecific) of the advertisement calls and associated morphological structures, this group should serve as an ideal model system for the study of frog call evolution, especially now that a relatively detailed and complete phylogenetic estimate of the various species is available.

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Appendix A. Estimated parameters from maximum likelihood analysis

General time-reversible model (GTR) + gamma rate heterogeneity (Γ) + invariant sites (PINVAR):

Base frequencies: A: 0.347609, C: 0.212590, G: 0.149945, T: 0.289856.

Rate matrix: AC: 2.21659; AG: 8.16426; AT: 2.35934; CG: 0.91908; CT: 16.52561; GT: 1.00000.

Shape parameter for gamma distribution: 0.560832.

Number of categories for discrete gamma approximation: 4.

Proportion of invariant sites: 0.338644.

Log likelihood of best tree: −21812.64132.

Appendix B. Classification of new world *Rana*

Rana (Although we consider all of the groups below to be part of *Rana*, the limits of this clade are beyond the scope of this paper. Phylogenetic definition of *Rana* must await a world-wide phylogenetic study of these frogs).

I. *Laurasiarana*, new clade name. Definition: The clade stemming from the most recent common ancestor of *Rana temporaria* Linne 1758 and *Rana aurora* Baird and Girard 1852. Etymology: From the name of the supercontinent of Laurasia, and the Latin word *rana*, meaning "frog," in reference to the distribution of this clade of frogs in North America, Europe, and Asia. Type species: *Rana aurora* Baird and Girard 1852.

A. Unnamed clade. This group was called the subgenus *Rana* (within the genus *Rana*) by Dubois (1992), but under phylogenetic nomenclature, one name cannot be applied to two different clades. Because there has been no phylogenetic definition of *Rana* to date, the name could be defined to refer to this clade. However, we prefer to use *Rana* for the more inclusive group of frogs. Dubois (1992) considered this clade to include 27 species in the *R. arvalis*, *R. chensinensis*, *R. graeca*, *R. japonica*, *R. tagoi*, and *R. temporaria* groups (he included *R. sylvatica* in the latter). Here, *Rana sylvatica* is explicitly excluded from this group, and we have collected data only on *R. temporaria* from this clade.

B. *Amerana* Dubois 1992 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana aurora* Baird and Girard 1852, *Rana boylii* Baird 1854, *Rana cascadae* Slater 1939, *Rana muscosa* Camp 1917, and *Rana pretiosa* Baird and Girard 1853. Content: Includes the species designated in the definition, as well as *Rana luteiventris* Thompson 1913 (if that taxon is considered a separate species from *Rana pretiosa* Baird and Girard 1852), and *Rana draytoni* Baird and Girard 1852 (if that taxon is considered a separate species from *Rana aurora* Baird and Girard 1852). This clade has been informally termed the *R. boylii* group by previous authors. Comments: Dubois (1992) named the section *Amerana*, which included two new subgenera: *Amerana* (including the species *R. boylii* and *R. muscosa*) and *Aurorana* (including the species *R. aurora*, *R. cascadae*, *R. draytoni*, and *R. pretiosa*). By our analysis (Fig. 1), all three of these names apply to the same clade, if the groups are made monophyletic.

II. *Novirana*, new clade name. Definition: The clade stemming from the most recent common ancestor of *Rana catesbeiana* Shaw 1802 and *Rana pipiens* Schreber 1782. Etymology: From the Latin words *novus*, meaning "new," and *rana*, meaning "frog," in reference to the New World distribution of this clade. Content: Includes *Rana sylvatica*, and all the species in *Aquarana* and *Sierrana* (see below). Type species: *Rana pipiens* Schreber 1782.

A. *Rana sylvatica* Le Conte 1825. Our sequence data place this species as the sister species to *Aquarana*. However, given the conflicting results between our data and previous analyses, we have not named the clade that includes *Rana sylvatica* and *Aquarana*.

B. *Aquarana* Dubois 1992 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana catesbeiana* Shaw 1802, *Rana clamitans* Latreille 1802, *Rana grylio* Stejneger 1901, *Rana heckscheri* Wright 1924, *Rana okaloosae* Moler 1985, *Rana septentrionalis* Baird 1854, and *Rana virgatipes* Cope1891. The species used in the definition are those species that were included by Dubois within this group. Content: Includes the species listed as specifiers in the definition. This clade has been informally termed the *R. catesbeiana* group by previous authors.

C. *Sierrana* Dubois 1992 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana juliani* Hillis and de Sá, 1988, *Rana maculata* Brocchi 1877, and *Rana sierramadrensis* Taylor 1939. Content: Includes all the species in *Ranula*, *Torrentirana*, and *Pantherana* (see below). Comments: This clade was named by Dubois as a subgenus; he specified the content as including the species that are used as specifiers in the definition. Note that this definition (based on the content specified by Dubois) results a much larger group than that envisioned by Dubois (1992).

1. *Ranula* Peters 1859 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana palmipes* Spix 1824 and *Rana warszewitschii* (Schmidt) 1857. Content: Includes the species in *Levirana* and *Lithobates* (see below). Comments: This clade was consistently recognized by E. D. Cope (as a genus) from 1866 until his death; he considered it to be the American counterpart of *Hylarana* Cope (1866). This group has been informally termed the *R. palmipes* group by previous authors.

a. *Levirana* Cope 1894 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana maculata* Brocchi 1877 and *Rana vibicaria* (Cope) 1894. Content: In addition to the species specified in the definition, this clade also includes *R. warszewitschii*.

i. *Rana maculata* Brocchi 1877.

ii. *Trypheropsis* Cope 1868 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana warszewitschii* (Schmidt) 1857 and *Rana vibicaria* (Cope) 1894. Content: Includes the species specified in the definition.

b. *Lithobates* Fitzinger 1843 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana palmipes* Spix 1824, *Rana vaillanti* Brocchi 1877, *Rana bwana* Hillis and de Sá 1988, and *Rana juliani* Hillis and de Sá 1988. Content: In addition to the species specified in the definition, includes *Rana gollmeri* (Peters) 1859 if that species is recognized as distinct from *R. palmipes*, and an undescribed species from northwestern Ecuador and western Colombia (species 1 in Figs. 1, 2).

2. *Torrentirana*, new clade name. Definition: The clade stemming from the most recent common ancestor of *Rana tarahumarae* Boulenger 1917 and *Rana sierramadrensis* Taylor 1939. Etymology: From the Latin words *torrentis*, referring to a swift or violent stream, and *rana*, meaning "frog," in reference to the typical habitat of many of the species in this clade. Content: Includes *Rana sierramadrensis* and the species within *Zweifelia* (see below). Type species: *Rana tarahumarae* Boulenger 1917.
   a. *Rana sierramadrensis* Taylor 1939.
   b. *Zweifelia* Dubois 1992 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana tarahumarae* Boulenger 1917 and *Rana zweifeli* Hillis, Frost and Webb 1984. Content: This clade includes the species named in the definition, as well as *Rana johni* Blair 1965, *Rana pueblae* Zweifel 1955, *Rana pustulosa* Boulenger 1883, and *Rana psilonota* Webb, 2001. At least two of the species (*R. johni* and *R. pueblae*) are thought to be extinct, and at least one of the remaining species (*R. tarahumarae*) is extinct over much of its former range. This clade has been informally termed the *R. tarahumarae* group by previous authors.
3. *Pantherana* Dubois 1992 (converted clade name). Definition: The clade stemming from the most recent common ancestor of *Rana pipiens* Schreber 1782, *Rana montezumae* Baird 1854, *Rana palustris* Le Conte 1825, and *Rana berlandieri* Baird 1854. Content: All of the species in *Stertirana*, *Nenirana*, and *Scurrilirana* (see below). This clade has been informally termed the *R. pipiens* complex by previous authors.
   a. *Stertirana*, new clade name. Definition: The clade stemming from the most recent common ancestor of *Rana pipiens* Schreber 1782 and *Rana montezumae* Baird 1854. Etymology: From the Latin words *sterto*, meaning "snore," and *rana*, meaning "frog," in reference to the snore-like element of the advertisement call of the frogs in this group. Content: Includes *R. pipiens* and the species in *Lacusirana* (see below). Type species: *Rana montezumae* Baird 1854.
      i. *Rana pipiens* Schreber 1782.
      ii. *Lacusirana*, new clade name. Definition: The clade stemming from the most recent common ancestor of *Rana montezumae* Baird 1854, *R. megapoda* Taylor 1942, and *Rana chiricahuensis* Platz and Mecham 1979. Etymology: From the Latin words *lacus*, meaning "lake," and *rana*, meaning "frog," in reference to the habitat of most of the species in this group. Content: In addition to the species named in the definition, this clade contains *Rana dunni* Zweifel 1957, *Rana megapoda* Taylor 1942, *Rana subaquavocalis* Platz 1993, *Rana lemosespinali* Smith and Chiszar 2003, and a least one undescribed species from the Mexican Plateau (species 2 in Figs. 1, 2). In addition, we place *Rana fisheri* Stejneger 1893 in this clade based on morphological similarity. Type species: *R. megapoda* Taylor 1942.
   b. *Nenirana*, new clade name. Definition: The clade stemming from the most recent common ancestor of *Rana areolata* Baird and Girard 1852 and *Rana palustris* Le Conte 1825. Etymology: From the Latin words *nenia*, meaning "a funeral song," and *rana*, meaning "frog," in reference to the low, mournful advertisement call of the species in this clade. Content: In addition to the species specified in the definition, this clade includes *Rana capito* LeConte 1855 and *Rana sevosa* Goin and Netting 1940. Hillis et al. (1983) informally termed this clade the *R. areolata* group. Type species: *Rana areolata* Baird and Girard 1852.
   c. *Scurrilirana*, new clade name. Definition: The clade stemming from the most recent common ancestor of *Rana berlandieri* Baird 1854, *Rana sphenocephala* Cope 1886, *Rana forreri* Boulenger 1883, *R. spectabilis* Hillis and Frost 1985, *Rana omiltemana* Gunther 1900, *Rana taylori* Smith 1959, and *Rana magnaocularis* Frost and Bagnara 1976. Etymology: From the Latin words *scurrilis*, meaning "jesting," and *rana*, meaning "frog," in reference to the advertisement calls of most of the species in this clade, which sound like chuckling laughter. Content: In addition to the species specified in the definition, this clade includes *Rana blairi* Mecham, Littlejohn, Oldham, Brown, and Brown 1973 *Rana chichicuahutla* Cuellar, Méndez-DeLaCruz, and Villagrán-Santa Cruz 1996, *Rana macroglossa* Brocchi 1877, *Rana miadis* Barbour and Loveridge 1929, *Rana neovolcanica* Hillis and Frost 1985, *Rana onca* Cope in Yarrow 1875, *Rana tlaloci* Hillis and Frost 1985, *Rana yavapaiensis* Platz and Frost 1984, and several undescribed species (species 3–8) in Mexico and Central America. Hillis et al. (1983) informally termed this clade the beta division of the *R. pipiens* complex. Type species: *Rana berlandieri* Baird 1854.

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