THE HISTORY OF A NEARCTIC COLONIZATION: MOLECULAR PHYLOGENETICS AND BIOGEOGRAPHY OF THE NEARCTIC TOADS (*BUFO*)

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Abstract.—Previous hypotheses of phylogenetic relationships among Nearctic toads (Bufonidae) and their congeners suggest contradictory biogeographic histories. These hypotheses argue that the Nearctic *Bufo* are: (1) a polyphyletic assemblage resulting from multiple colonizations from Africa; (2) a paraphyletic assemblage resulting from a single colonization event from South America with subsequent dispersal into Eurasia; or (3) a monophyletic group derived from the Neotropics. We obtained approximately 2.5 kb of mitochondrial DNA sequence data for the 12S, 16S, and intervening valine tRNA gene from 82 individuals representing 56 species and used parametric bootstrapping to test hypotheses of the biogeographic history of the Nearctic *Bufo*. We find that the Nearctic species of *Bufo* are monophyletic and nested within a large clade of New World *Bufo* to the exclusion of Eurasian and African taxa. This suggests that Nearctic *Bufo* result from a single colonization from the Neotropics. More generally, we demonstrate the utility of parametric bootstrapping for testing alternative biogeographic hypotheses. Through parametric bootstrapping, we refute several previously published biogeographic hypotheses regarding *Bufo*. These previous studies may have been influenced by homoplasy in osteological characters. Given the Neotropical origin for Nearctic *Bufo*, we examine current distributional patterns to assess whether the Nearctic-Neotropical boundary is a broad transition zone or a narrow boundary. We also survey fossil and paleogeographic evidence to examine potential Tertiary and Cretaceous dispersal routes, including the Paleocene Isthmian Link, the Antillean and Aves Ridges, and the current Central American Land Bridge, that may have allowed colonization of the Nearctic.

Key words.—Bayesian analysis, biogeography, heteroplasmy, hypothesis testing, mitochondrial ribosomal DNA, parametric bootstrapping.

Congruent distributions of organisms have been used to infer broad, general patterns of biogeography (Sclater 1858; Wallace 1876; Rosen 1978; Wiley 1988). The identification of such patterns allows comparative biologists to test general hypotheses of the origin, vicariance, and dispersal of biotas. During the descriptive phase of biogeography, congruent distributions led to the recognition of six major biogeographic regions (Sclater 1858; Wallace 1876). Faunal differences at the boundaries between regions, such as the transition from the Australian region's marsupials and megapodes to the Oriental region's placental mammals and woodpeckers at Wallace's Line, are due to restricted dispersal. In contrast are taxa with distributions in multiple regions. However, the phylogenetic relationships among the constituent species of these more cosmopolitan taxa may still reflect the limited dispersal associated with regional boundaries.

Biogeography has recently entered a hypothesis-testing phase in which shared distributional patterns can be tested for congruence so that general patterns can be elucidated. Phylogenetic studies of widespread groups that cross regional boundaries are ideal for testing the efficacy of boundaries as barriers to dispersal (e.g., Evans et al. 2003). Moreover, in combination with distributional data, the phylogenetic data add a historical component that permits exploration of two fundamental questions in biogeography: how biogeographic regions were colonized and how regional faunas evolved (Cracraft 1988, 1994).

Here, we use a phylogenetic approach with hypothesis testing to address several long-standing controversies about the history of colonization of the Nearctic region (Greenland, Canada, the United States, and the Central Highlands of Mexico) by toads (*Bufo*). *Bufo* is a nearly cosmopolitan genus, with representatives in all six biogeographic regions (following Wallace 1876), which is the largest distribution of any amphibian genus. Toads are a major component of the Nearctic frog fauna, with 31% of species. Within Bufonidae, 33 genera are recognized, but more than half of the approximately 450 species are *Bufo* (Frost 2002). All but one of the non-*Bufo* genera in this family contain fewer than 22 species, and all are isolated to a single biogeographic region (Frost 2002). Evidence suggests, however, that *Bufo* is not monophyletic, and there are no synapomorphies uniting the genus (Graybeal and Cannatella 1995). Nevertheless, the cosmopolitan distribution is not just an artifact of taxonomy; instead, the taxonomic uncertainties result from the phenotypic and ecological homogeneity of this widespread taxon, which further counters the morphological distinctness and endemicity expected within biogeographic regions.

This unique diversity, distribution, and taxonomic uncertainty has prompted numerous systematic and biogeographic investigations of toads, especially the Nearctic *Bufo*. Three distinct biogeographic hypotheses, involving various intercontinental dispersal events, exist for the origin of the Nearctic toads. Based on osteology, Tihen (1962a) argued for an African origin for *Bufo* with the Nearctic species consisting of three lineages. He argued that one of these lineages is the sister taxon to the Eurasian *B. bufo*, and another Nearctic lineage led to all Middle American and many South American toads (Fig. 1a). Therefore, this hypothesis suggests the Nearctic *Bufo* are polyphyletic (Nearctic polyphyly hypothesis). Alternatively, Blair (1972a) suggested a South American origin for the genus with the Nearctic *Bufo* as part
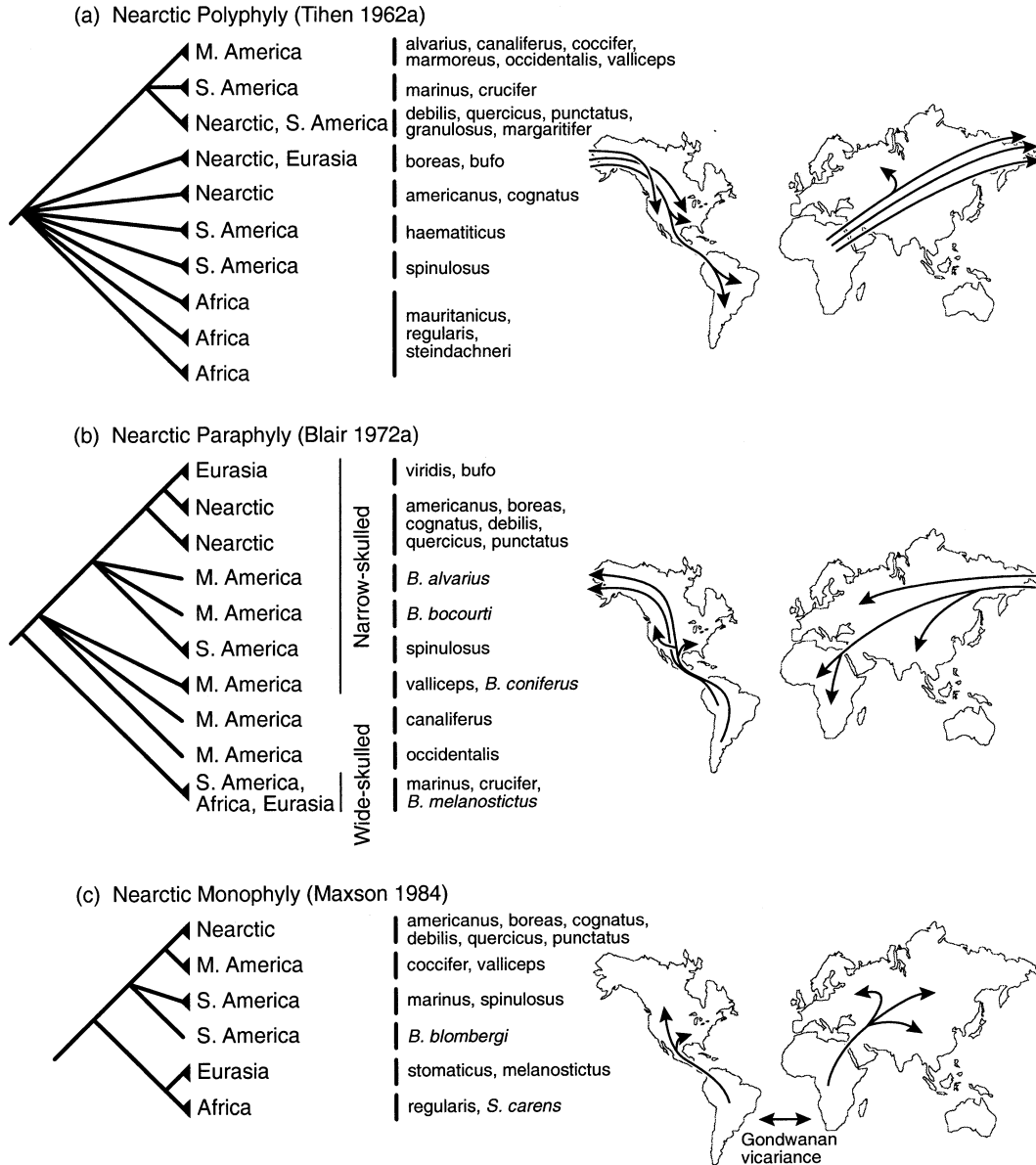
FIG. 1.   Tree topologies and potential dispersal/colonization routes representative of previously proposed biogeographic hypotheses for the origin of the Nearctic *Bufo*. Species groups and species (italicized) listed to the right of each topology represent the taxa sampled in each study. (a) Nearctic polyphyly hypothesis (Tihen 1962a). Although Beringian dispersal is indicated on this map, Tihen (1962a) never hypothesized whether Nearctic colonization was Beringian or trans-Atlantic. Also, note that the colonization routes for the South American *B. haematiticus* and *B. spinulosus* are not depicted because they are not described in sufficient detail by Tihen (1962a). (b) Nearctic paraphyly hypothesis (Blair 1972a). Colonization routes of the Middle American taxa basal to the narrow-skulled group are not indicated due to lack of sufficient information. (c) Nearctic monophyly hypothesis (Maxson 1984). The Nearctic taxa listed are from Maxson et al. (1981) and Maxson (1984), and the phylogeny depicts information from the text and figures of these articles. Maps modified after Blair (1972a, fig. 18–1).

of a narrow-skulled lineage that included some South American, Middle American, and Eurasian taxa (Fig. 1b). Other South American and Eurasian toads were placed in a wide-skulled lineage that may have also used the Nearctic and Beringia as a colonization route but failed to leave any extant Nearctic descendants. Moreover, he argued that the narrow-skulled Eurasian taxa descended from Nearctic ancestors, which suggests the Nearctic *Bufo* are paraphyletic (Nearctic paraphyly hypothesis). This interpretation was based largely on osteological characters (R. F. Martin 1972), but morpho-

logical, cytological, biochemical, genetic, and vocal characters were also considered (Blair 1972b,c; Bogart 1972; Cei et al. 1972; Guttman 1972; Low 1972; R. F. Martin 1972; W. F. Martin 1972; Szarski 1972). Finally, following studies of albumin evolution (Maxson 1981a,b, 1984; Maxson et al. 1981), Maxson (1984) concluded that the Nearctic *Bufo* are monophyletic and that together with the Middle American *Bufo*, represent a single northward radiation from a South American ancestor without any Eurasian or Old World descendants (Nearctic monophyly hypothesis; Fig. 1c). Addi-

tionally, in contrast to Blair (1972a), Maxson suggested the origin of *Bufo* occurred in western Gondwana rather than strictly in South America.

Recent studies have also generated conflicting phylogenetic results regarding the Nearctic *Bufo*. Mitochondrial DNA (mtDNA) analyses included several results consistent with the Nearctic paraphyly hypothesis, such as the nesting of some Eurasian taxa within the Nearctic *Bufo* (Goebel 1996; Graybeal 1997), although neither study supported the recognition of wide- and narrow-skulled clades. However, Graybeal's (1997, fig. 13) combined analysis of morphological and mtDNA data suggested monophyly of the Nearctic *Bufo*. Relationships among other New World *Bufo*, including nonmonophyly of the North American taxa (Nearctic and Middle American), were not consistent with the Nearctic monophyly hypothesis. We do not treat either Goebel's (1996) or Graybeal's (1997) phylogenies as unique biogeographic hypotheses because Goebel's (1996) hypothesis is only a minor variant of the Nearctic paraphyly hypothesis and Graybeal's (1997) combined results for the Nearctic taxa are consistent with the Nearctic monophyly hypothesis. The biogeographic implications of Graybeal's (1997) results for the non-Nearctic *Bufo* are difficult to interpret because they suggest a large number of intercontinental dispersal events between South America, Africa, and Eurasia.

None of these hypotheses specifically argues for a time of colonization or associates colonization of the Nearctic with specific events in the geologic record. Savage (1966, 1973), however, suggested that *Bufo*, in addition to several other anuran groups, entered the Nearctic via the Isthmian Link, a proposed Paleocene (about 58–65 million years ago) Central American Land Bridge. Although this scenario was originally described in accordance with Blair's (1972a) interpretation of *Bufo* relationships, it provides a mode of dispersal consistent with both the Nearctic paraphyly and Nearctic monophyly hypotheses.

Due to these conflicting results, the history of Nearctic colonization by *Bufo* and the relationships between the Nearctic species groups and other *Bufo* remain uncertain. In this study, we use intensive taxon sampling and phylogenetic analyses to address these issues. Because the major hypotheses for Nearctic colonization can be differentiated by their unique predictions about whether the Nearctic *Bufo* form a polyphyletic, paraphyletic, or monophyletic group, we use statistical hypothesis testing to discriminate among them. Additionally, we use fossil and paleogeographic evidence to address potential colonization routes. In combination, these approaches allow us to interpret the evolutionary origin and biogeographic history of the Nearctic *Bufo*.

MATERIALS AND METHODS

The characterization of a taxon as either Nearctic, Middle American, or South American (Appendix 1) is based on it either inhabiting that region or being part of a clade in which the majority of members inhabit the region, and any extra-regional members result from secondary colonization events. As with the Nearctic, biogeographic definitions can be applied to South America and Middle America; they correspond to Wallace's (1876) subregions 1 and 2 and subregion 3 of the Neotropical region, respectively. Here we use North America to mean Middle America plus the Nearctic.

Taxon Sampling

Samples from 82 specimens representing 56 species were analyzed, including 78 *Bufo*, two non-*Bufo* bufonids, and four hyloids (Appendix 1). The hyloids were specified as the outgroup taxa and were chosen based on the results of Darst and Cannatella (2004). The monophyly of the ingroup, Bufonidae, is well supported (Ford and Cannatella 1993). For most polytypic and/or geographically widespread Nearctic species, sampling incorporated subspecific and geographic diversity. Also, the inclusion of multiple individuals per species assists alignment and confirmation of sequence identity. Representatives of all currently recognized Nearctic *Bufo* taxa (species and subspecies) were included except for *B. mexicanus*, *B. kelloggi*, and *B. compactilis* (Appendix 1). At least one representative of each Middle American and South American species group (following Blair 1972d, appendix A; Cei 1972; R. F. Martin 1972) was also included except for the high-elevation, narrowly distributed *B. periglenes* and *B. holdridgei*. Eurasian sampling included five of the six recognized species groups (following Inger 1972). Tihen (1962a) postulated a relationship between extant Nearctic toads and African *B. regularis*–like ancestors. The sampling of African *Bufo* includes representatives of the *B. regularis* group and two taxa outside of this lineage to insure that we captured all potential *B. regularis* group lineages. Therefore, representatives of each species or species group of South American, Middle American, Eurasian, and African *Bufo* that were described as potential ancestors or descendants of Nearctic *Bufo* in the aforementioned hypotheses are sampled.

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted from liver or muscle tissue with the DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA). Overlapping sets of primers were used to amplify approximately 2.5 kb of the mitochondrial 12S and 16S genes and the intervening valine tRNA (Table 1). This region corresponds to positions 2153–4574 in the complete mitochondrial sequence of *Xenopus laevis* (GenBank reference sequence NC-001573 derived from M10217; the reference sequence and our counts do not include 142 bases that occur in other anurans, including other *Xenopus* [e.g., Genbank Y10943] that would be between 2228 and 2229 in the reference sequence). Amplification followed standard polymerase chain reaction conditions (Palumbi 1996) with the following thermal cycle profile: 2 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 46°C for 30 sec, and 72°C for 60 sec, and a final extension phase at 72°C for 7 min. Amplified products were purified from 1% agarose gel slices using QIAquick Gel Extraction Kits (Qiagen, Inc.). Cycle sequencing reactions were completed with ABI Prism BigDye Terminator chemistry (Ver. 2 and 3; Applied Biosystems, Foster City, CA), purified with Sephadex G-50 (S-6022 Sigma, St. Louis, MO) in Centrisep columns (CS-901 Princeton Separations, Princeton, NJ), and analyzed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Editing and assembly of

TABLE 1. Primers used for amplification and/or sequencing. Position is relative to *Xenopus laevis* (GenBank accession NC 001573). Goebel refers to primers listed in Table 3 of Goebel et al. (1999). All other primers designed in the labs of D. M. Hillis and D. C. Cannatella, including modified versions of primers listed in Goebel et al. (1999).

Primer name	Position	Primer sequence (5′ to 3′)	Goebel
MVZ59[1]	2157–2180	ATAGCACTGAAAAYGCTDAGATG	29
12L1[2]	2475–2509	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	46
12Sar-H	2486–2509	ATAGTGGGGTATCTAATCCCAGTT	51
12Sm[1]	2968–2988	GGCAAGTCGTAACATGGTAAG	
tRNA-val[2]	3034–3059	GGTGTAAGCGARAGGCTTTKGTTAAG	73
MVZ50[1]	3042–3063	TCTCGGTGTAAGCGAGAGGCTT	72
16Sh[1]	3282–3304	GCTAGACCATKATGCAAAAGGTA	76
16Shr[3]	3282–3304	TACCTTTTGCATMATGGTCTAGC	
16Sc[1]	3623–3642	GTRGGCCTAAAAGCAGCCAC	
16Sa[1]	3956–3975	ATGTTTTTGGTAAACAGGCG	87
16Sd[1]	4549–4574	CTCCGGTCTGAACTCAGATCACGTAG	

[1] Primary primers used.
[2] Secondary primers used for only a fraction of the individuals.
[3] Used only for *Bufo baxteri*.

contigs was completed using Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI).

Alignment and Phylogenetic Analyses

Initial alignment of DNA sequences was completed in ClustalX (Thompson et al. 1997). Manual adjustments were then made in MacClade 4.0 (Maddison and Maddison 2000) so as to minimize the number of changes required across sites. Autapomorphies were verified by examining the chromatograms, and secondary structure models were examined to aid in aligning regions that were otherwise ambiguous (Cannone et al. 2002). Aligned sequences were analyzed using PAUP* (Ver. 4.0b10; Swofford 2000). Parsimony analyses were conducted using a heuristic search with 1000 random addition-sequence replicates and TBR branch swapping. Nodal support was assessed through nonparametric bootstrap analysis using 1000 bootstrap replicates with 10 random addition-sequence replicates per bootstrap replicate.

The most appropriate model of evolution for the likelihood analysis was estimated through likelihood-ratio tests of the complete sequence (12S, tRNA-Val, and 16S) using Modeltest 3.06 (Posada and Crandall 1998). Likelihood analysis was conducted through successive iterations with starting parameters based on estimates from the previous result. Parameters for the first iteration were estimated from the most-parsimonious tree with the best likelihood score. For computational efficiency, the first few searches were conducted with branch length optimization parameters set at a pass limit of 10 and smoothing passes were stopped when the likelihood score changed (delta value) by less than 10^{-5} likelihood units. Once an iteration yielded a tree score equal to or less than the previous iteration, the optimization parameters were set to more stringent values (pass limit = 20; delta = 10^{-6}). Iterations were continued until successive searches yielded identical trees. To prevent searching of highly nonoptimal topologies and thereby improve computational efficiency, a constraint tree was used. This tree constrained most apical nodes with 95% or greater bootstrap support in the parsimony analysis to be monophyletic (see Fig. 3). All constrained nodes were either within a species or among closely related

taxa. Strongly supported nodes that were inconsistent with traditional relationships were not constrained.

Four replicate Bayesian analyses were conducted with MrBayes 3.04b (Huelsenbeck and Ronquist 2001) on an NPACI Rocks cluster (http://www.rockscluster.org). Four Markov chains were used in each replicate, and the chain was sampled every 100 generations. The temperature parameter was set to 0.3 and proposal parameters were tuned to improve acceptance and sampling efficiency. Analyses were allowed to run for 20 million generations.

Hypothesis Testing

Parametric bootstrapping was used to test hypotheses regarding the evolutionary history of the Nearctic *Bufo*. The question is whether a dataset can reject a given null hypothesis such as the monophyly of the Nearctic toads and the Eurasian *B. bufo* as suggested in the Nearctic paraphyly hypothesis. Parametric bootstrapping involves using simulation to determine the probability that the observed relationships result from an evolutionary history consistent with a specified null hypothesis (Hillis et al. 1996; Huelsenbeck et al. 1996a,b; termed the SOWH test by Goldman et al. 2000). Parameters estimated from the observed data and a phylogeny consistent with the null hypothesis being tested (found using a constraint tree) are used to simulate replicate datasets. These datasets can then be analyzed for the best tree overall and the best tree consistent with the null hypothesis to create a distribution of tree length/score differences. This value can also be obtained for the observed data. By comparing the observed value to the distribution of expected values, a null hypothesis can be rejected as the underlying evolutionary history if the observed difference is greater than 95% of the expected differences (assuming the critical value for alpha, the probability of a Type I error, is 0.05).

For some hypotheses, not all taxa were sufficiently discussed in the literature to allow for placement in a constraint tree and were excluded from hypothesis testing. Additionally, eight individuals in the *B. americanus* group with closely related sequences were removed to improve computational efficiency (see Appendix 2). Constraint trees are either de-

scribed in the Results or provided in Appendix 2. Modeltest 3.06 (Posada and Crandall 1998) was used to determine the most appropriate model of sequence evolution for each reduced-taxon dataset. Likelihood parameters were estimated from the most-parsimonious tree compatible with the constraint (null) tree and used to simulate 1000 replicate datasets. If multiple most-parsimonious trees were recovered, the one with the best likelihood score was selected. For each replicate dataset, two parsimony heuristic searches were conducted with 100 random addition-sequence replicates and TBR branch swapping. One search was used to find the unconstrained optimal tree, and the second was used to find the optimal tree consistent with the constraint. The difference in tree length between these two trees for each replicate dataset was used to construct the expected distribution.

We used parsimony as the optimality criterion for analysis of the simulated datasets because the computational requirements for examining datasets with likelihood are excessive given the number of parametric bootstrap tests we conducted. This approach may also reduce potential overconfidence in the parametric bootstrapping results. Parametric bootstrapping can suffer from Type I error if the assumed model used to simulate the datasets consistent with the null hypothesis deviates too much from the actual model of sequence evolution (Huelsenbeck et al. 1996b; Buckley 2002). One source of overconfidence, potentially leading to Type I error, is the perfect fit of the models used to generate and analyze each replicate dataset, which results in likelihood ratio values close to zero (Huelsenbeck et al. 1996b; Buckley 2002). Because this perfect fit is rarely matched in datasets from real organisms, Buckley (2002) suggested that a more realistic and less discriminatory approach may be to use a parameter-rich model for generating datasets that are then analyzed under parsimony. This suggestion is supported by the results of Sullivan et al. (2000, table 2), but an explicit power analysis has never been conducted.

Character-State Reconstruction

Character data were obtained from R. F. Martin (1972) for skull or frontoparietal type and from Blair (1972a,e) for lineage type. Species were categorized as narrow-, intermediate-, or wide-skulled following R. F. Martin's (1973) classifications based upon the width of the frontoparietal and the extensiveness of skull ossification. Blair (1972c) assigned species to narrow-, intermediate-, or wide-skulled lineages based in part on osteological data but also on numerous other characters; we followed Blair's categorization. Character evolution was inferred by mapping character states onto a condensed version of the maximum-likelihood topology using MacClade 4.0 (Maddison and Maddison 2000). This tree resulted from removing conspecific terminals and reducing the African clade to a single terminal because all members were assigned to the same lineage and skull type. Additional taxa were excluded if there was insufficient information to assign both character states.

RESULTS

Sequence Variation and Alignment

The DNA sequences are deposited in GenBank (Appendix 1) and the final alignment is deposited in TreeBASE. In the final alignment, positional homology was ambiguous for eight regions totaling 128 bases. These regions correspond to the following positions in *X. laevis* (GenBank accession NC 001573): 2930–2935; 3112–3116; 3123–3153; 3496–3528; 3606–3615; 3672–3684; 4247–4260; 4331–4340. Comparisons to secondary structure and a large dataset of nonbufonid hyloids (Darst and Cannatella 2004; D. C. Cannatella and D. M. Hillis, unpubl. data) were not informative in resolving the alignment of these regions, and they were excluded from analysis. In these regions, however, homology was recognizable among all or most of the ingroup taxa. Because exclusion of entire regions for all taxa discards useful information, these regions were examined for support for particular apical relationships.

In the final alignment, 1036 of the 2370 nucleotide positions were variable and 730 were parsimony informative. Corrected pairwise sequence divergence between all *Bufo* and the outgroup taxa ranged from 0.27 to 0.74 substitutions/site (0.27–0.48 substitutions/site with *Eleutherodactylus w-nigrum* excluded) and ranged up to 0.24 substitutions/site within *Bufo*. The large sequence divergence between *E. w-nigrum* and other taxa was expected based on sequences of other *Eleutherodactylus* (Darst and Cannatella 2004). The sequences of *B. canorus* (MVZ 142987) and *B. nelsoni* (MVZ 142829) were identical, as were those of two *B. microscaphus* (USNM 311161 and MVZ 223282); one of each pair was excluded from analysis.

Heteroplasmy

Two apparent cases of heteroplasmy, or multiple different mitochondrial genomes in an individual, were detected. In the first, a G-to-A transition occurs in *B. coniferus* at position 2893 of *X. laevis* (GenBank NC-001573). This position is highly conserved across all anurans (D. C. Cannatella and D. M. Hillis, unpubl. data). Based on secondary structure models for *X. laevis* (Cannone et al. 2002), this transition occurs in a stem region with G-T (U) pairing in all *Bufo* sampled. The observed transition establishes Watson-Crick pairing in the novel sequence. We used the ambiguity code R for scoring this base. The second case is a deletion of an A and a G in *B. baxteri* between positions 3267 and 3270. Because the ancestral sequence is 5′-AGAG-3′, the position of the deletion event is ambiguous. Based upon secondary structure models, this region is the start site of a small stem and loop, with the 5′ stem region consisting of the three bases GAG. Therefore, in the novel *B. baxteri* sequence, at least one of the pairing bases of this stem is lost. Because this region is invariant across the *B. americanus* group, we only analyzed the sequence without the deletion. In both cases, several extractions with multiple amplifications and sequencing using a variety of primer pairs were used to rule out contamination. A nuclear pseudogene is extremely unlikely to account for the multiple copies because only the aforementioned sites within the 2.5 kb examined were affected. Goebel (1996) and Goebel et al. (1999) also reported heteroplasmy in *Bufo* mitochondrial sequences.

Phylogenetic Relationships

The maximum-likelihood analysis used a GTR $+$ Γ $+$ I model of evolution and included five iterations of which the

last two incorporated the more demanding pass limit and delta value (see Materials and Methods). To improve computational efficiency, 17 nodes were constrained to be monophyletic; corrected sequence divergence among members of these clades was always less than 0.05 substitutions/site. The likelihood score of our final tree is 24571.7486 (estimated base frequencies: A: 0.3720, C: 0.2122, G: 0.1503, T: 0.2655; rate matrix: A-C: 6.2089, A-G: 19.9840, A-T: 8.8977, C-G: 0.5064, C-T: 57.3152, G-T: 1.0000; shape parameter for gamma distribution: 0.5476; proportion of invariant sites: 0.4348). Maximum-parsimony analysis generated six most-parsimonious trees of 5008 steps (CI = 0.336, RI = 0.624).

For the Bayesian analysis, plots of model parameters and likelihood versus generation number suggested that stationarity was reached by 150,000 generations. However, bipartition posterior estimates obtained from all samples after burn-in did not appear to converge in pairwise comparisons between runs (using the comparetree command in Mr Bayes and an arbitrarily chosen threshold value of <10% differences in posterior probability for the same bipartition) until the burn-in had been increased to 2–5 million generations. Therefore, we chose the conservative value of 5 million generations as the burn-in. Pairwise comparisons of bipartition posterior probability between independent runs after the burn-in was set to 5 million yielded similar values (differences <10%) among three runs. Comparisons to the fourth run were greater than the threshold value, and samples from this run were not included in the final pooled sample. Therefore, the last 15 million generations (i.e., 150,000 sampled trees) of these three runs were combined, yielding 450,000 trees for the final Bayesian posterior estimates. Bayesian posterior probabilities (bpp) of nodes recovered in the maximum-likelihood tree are shown in Figure 2.

Likelihood and parsimony analyses yielded very similar results. In all cases, the ingroup taxa (Bufonidae) form a well-supported clade, and *Osornophryne guacamayo* is the sister taxon to the remaining bufonids. As a result, only relationships among the ingroup taxa are shown. If the resulting trees differed among analyses, the differences are discussed if they have important phylogenetic or biogeographic implications.

The likelihood, Bayesian, and parsimony analyses revealed a clade that we term the New World clade. This clade includes three distinct clades, here termed the Nearctic clade, the Middle American clade, and the *B. marinus* clade (Figs. 2, 3). The New World clade does not include any Eurasian or African taxa, and several South American species are also excluded. Despite the exclusion of these South American species, we term it the New World clade because it is the largest *Bufo* radiation in the New World and includes all Middle American and Nearctic *Bufo* and a large number of the South American species.

Relationships among the Nearctic clade, Middle American clade, and *B. marinus* clade are not clearly resolved. The maximum-likelihood tree recovers the *B. marinus* clade as sister taxon of the Nearctic clade (bpp = 41 if the reconstruction of the South American *B.* cf. *margaritifer* is not evaluated; see Figs. 2, 4), but the Bayesian analysis weakly favors North American (Nearctic + Middle American) monophyly (bpp = 46).

All most-parsimonious trees recover the Nearctic clade and

the New World clade, although *B.* cf. *margaritifer* is reconstructed as outside of the latter (Fig. 3). Within the New World clade, a sister-group relationship between the *B. marinus* clade and the Nearctic clade is favored by parsimony, but as in the likelihood analysis, this relationship is weakly supported (nonparametric bootstrap support, npb = 36). The most-parsimonious trees suggest that the Middle American *B. bocourti* is the sister taxon to this clade (Nearctic clade + *B. marinus* clade). This relationship is only one step shorter than a tree with monophyly of the Middle American clade. Middle American monophyly is weakly supported (npb = 50), but other reconstructions of *B. bocourti* received even poorer support (npb ≤ 11). The content of and relationships among the South American lineages also differ from the likelihood results because *B.* cf. *margaritifer* is outside of the New World clade and not within the *B. marinus* clade. Nevertheless, like the Bayesian and likelihood analyses, the parsimony bootstrap analysis supports the recognition of three major New World clades.

In the likelihood and parsimony analyses, relationships among the Eurasian, African, and South American taxa outside of the New World clade are not well resolved except for an African clade (Figs. 2, 3). The African bufonid *Schismaderma carens* is nested within *Bufo*, a result consistent with several previous studies (Maxson 1981; Graybeal 1997). The South American *B. variegatus* and *B. haematiticus* are basal to all other *Bufo* in the maximum likelihood and four of the six most-parsimonious trees (*B. variegatus* is basal to all *Bufo* in all six most-parsimonious trees).

Nearctic clade.—The Nearctic *Bufo* are monophyletic (bpp = 100; npb = 74), and the *B. boreas* group is the sister taxon to the rest of the Nearctic clade (Fig. 2, 3). Within the *B. boreas* group, *B. boreas* and *B. canorus* are each not monophyletic, a result consistent with previous research (Goebel 1996; Graybeal 1993; Shaffer et al. 2000). The monophyly of each Nearctic species group is strongly supported (bpp = 100; npb = 100) except for the *B. cognatus* group. The best-supported reconstruction suggests that the *B. cognatus* group (*cognatus* and *speciosus*) is paraphyletic (bpp = 70; npb = 47). An alternative topology favoring monophyly of *B. cognatus* and *B. speciosus* is poorly supported (bpp = 9; npb = 40), but two synapomorphies supporting the monophyly of this group exist in the excluded ambiguous regions.

Nontraditional relationships were found among representatives of the *B. americanus* group. *Bufo woodhousii* is the sister taxon of a clade including *B. americanus*, *B. houstonensis*, and *B. velatus*. *Bufo terrestris* is nested within a paraphyletic sample of *B. fowleri* individuals. Masta et al. (2002) reported similar results. However, our geographic and taxonomic sampling reveals novel results including the monophyly of mitochondrial haplotypes of *B. americanus charlesmithi*, *B. houstonensis*, and *B. velatus* to the exclusion of *B. americanus americanus*.

Bufo marinus clade.—The South American *Bufo* form seven species groups (following Blair 1972d, appendix A; R. F. Martin 1972; but see Duellman and Schulte 1992; Pramuk 2002). In the likelihood analysis, *B. crucifer*, *B. marinus*, *B. granulosus*, *B. spinulosus*, and *B.* cf. *margaritifer*, which represent five groups, form a clade (bpp = 48) to the exclusion of *B. haematiticus* and *B. variegatus*, which represent the two

Fig. 2. Maximum likelihood topology. Numbers are the Bayesian posterior probabilities from 450,000 sampled trees. Bold numbers are Bayesian posterior probabilities if *Bufo* cf. *margaritifer* is excluded (see Results). Outgroup taxa are not shown. Branches in shaded boxes are drawn 10 times longer than those in the rest of the tree so that the resolution can be seen.

other groups (Appendix 1). Although no West Indian toads were sampled, these are presumably members of the *B. marinus* clade and thus part of this radiation (Pramuk 2002). The weak Bayesian support for the *B. marinus* clade and for the New World clade is due to *B.* cf. *margaritifer*, which in the parsimony analysis is outside of the New World clade. *Bufo* cf. *margaritifer* has a very divergent sequence, making accurate reconstruction difficult. Sequence divergence (GTR + Γ + I) between members of the *B. marinus* clade and *B.* cf. *margaritifer* is 0.155–0.194 substitutions/site as compared to only 0.171 substitutions/site between *B.* cf. *margaritifer* and the next most similar sequence, *B. bocourti*. Sequence divergence among all other members of the *B. marinus* clade does not exceed 0.09 substitutions/site. If *B.* cf. *margaritifer* is pruned from the population of trees used to generate the Bayesian consensus tree, then high support for the mono-
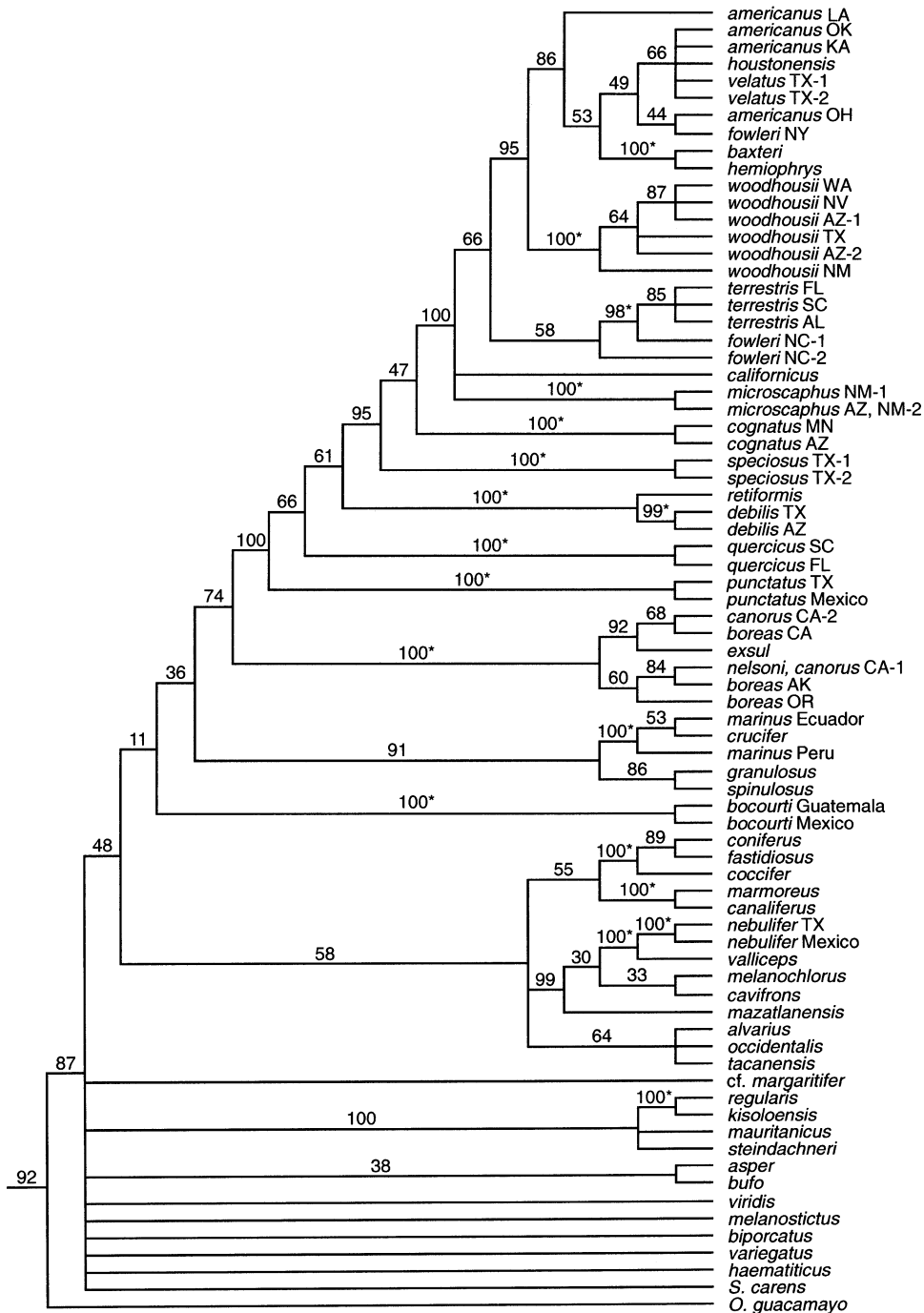
FIG. 3. Strict consensus of the six most parsimonious trees (CI = 0.336, RI = 0.624). Nonparametric bootstrap support values associated with each node are given as percentages of 1000 pseudoreplicates. An asterisk denotes clades constrained as monophyletic in the likelihood search (see Materials and Methods). Outgroup taxa are not shown.

phyly of the New World clade (bpp = 99) and the monophyly of the *B. marinus* clade (bpp = 100) results (Fig. 2).

Middle American clade.—In the likelihood analysis, the Middle American *Bufo* are monophyletic (bpp = 99), and *B. bocourti* is the sister taxon to the other Middle American taxa (Fig. 2). In the parsimony analysis, monophyly of the Middle American *Bufo* is weakly supported (npb = 50) as are alternate reconstructions of *B. bocourti*. The remaining Middle

American species form four clades: (1) the *Bufo valliceps* group including *B. valliceps*, *B. nebulifer*, *B. mazatlanensis*, *B. melanochlorus*, and *B. macrocristatus*; (2) *B. coniferus*, *B. fastidiosus*, and *B. ibarrai*; (3) *B. marmoreus* and *B. canaliferus*; and (4) *B. alvarius*, *B. occidentalis*, and *B. tacanensis*.

Relationships within the New World clade.—A complete interpretation of the biogeographic and evolutionary history of the Nearctic *Bufo* requires identifying its sister taxon (also
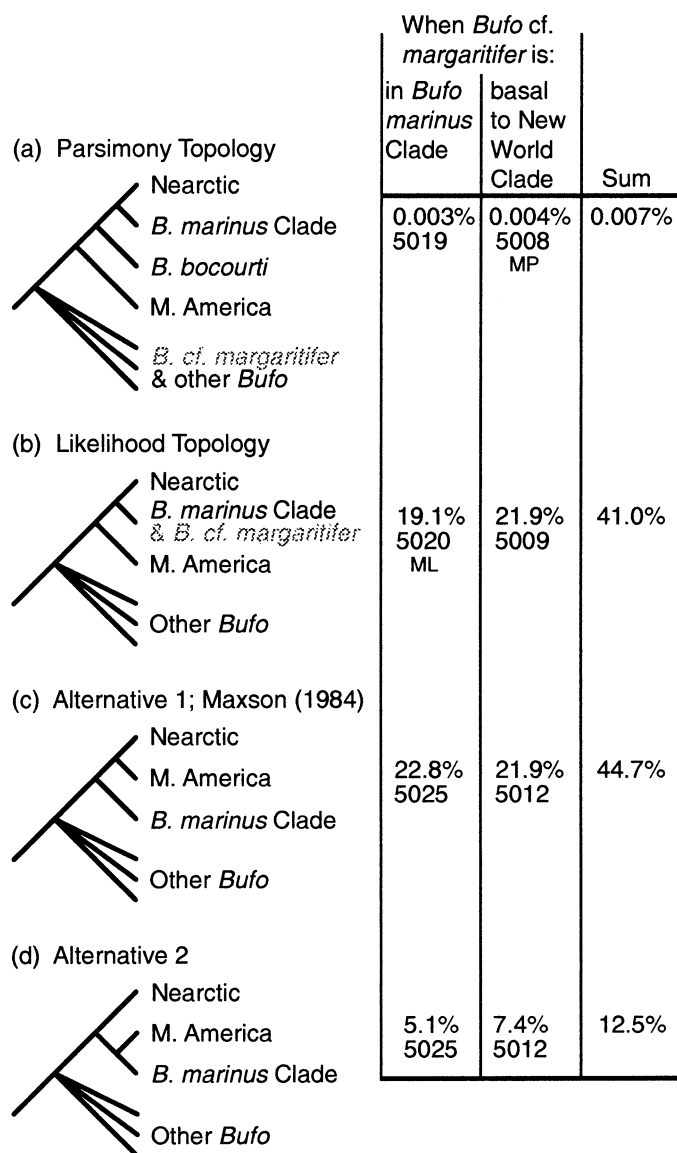
	When *Bufo* cf. *margaritifer* is:		
	in *Bufo marinus* Clade	basal to New World Clade	Sum
(a) Parsimony Topology			
Nearctic			
B. marinus Clade	0.003%	0.004%	0.007%
B. bocourti	5019	5008 MP	
M. America			
B. cf. margaritifer & other *Bufo*			
(b) Likelihood Topology			
Nearctic			
B. marinus Clade & *B. cf. margaritifer*	19.1%	21.9%	41.0%
M. America	5020 ML	5009	
Other *Bufo*			
(c) Alternative 1; Maxson (1984)			
Nearctic			
M. America	22.8%	21.9%	44.7%
B. marinus Clade	5025	5012	
Other *Bufo*			
(d) Alternative 2			
Nearctic			
M. America	5.1%	7.4%	12.5%
B. marinus Clade	5025	5012	
Other *Bufo*			

FIG. 4. Bayesian support and parsimony tree length of different topologies within the New World clade. Relationships among members of each of the three New World clades (the Nearctic, Middle American, and *B. marinus* clades) were not considered for determining similarity between the Bayesian and parsimony topologies. (a) The general structure of the maximum parsimony topology, (b) the maximum likelihood topology, (c) alternative topology consistent with Maxson's (1984) hypothesis, and (d) alternative topology reflecting the third possible reconstruction of the three clades in the New World clade. Placement of *Bufo* cf. *margaritifer* in the most parsimonious (MP) and maximum likelihood (ML) trees is shown in gray. The first and second columns list Bayesian posterior probabilities and tree lengths of topologies with alternative reconstruction of *B.* cf. *margaritifer*. The third column lists the sum of Bayesian posterior probabilities from columns 1 and 2, which is the total support for relationships among members of the New World clade regardless of the position of *B.* cf. *margaritifer*. Parsimony heuristic searches were conducted with 1000 random addition-sequence replicates and TBR branch swapping.

see Hypothesis Testing, below). Given that the Nearctic clade, Middle American clade, and *B. marinus* clade are each monophyletic, then there are three possible arrangements. Parsimony and likelihood analyses suggest (Nearctic clade + the *B. marinus* clade) (Fig. 4a,b), although the Bayesian analysis shows the greatest support for (Nearctic clade + Middle American clade) (Fig. 4c), which is consistent with Maxson's (1984) hypothesis. The third arrangement, (Nearctic clade (Middle American clade + the *B. marinus* clade)) (Fig. 4d), was not recovered in the parsimony and likelihood analyses.

We assessed the support for each of these by examining the lengths of the most-parsimonious trees and the sampling frequency in the Bayesian analysis (where sampling density is a function of likelihood score) of trees compatible with each hypothesized reconstruction. We examined relationships only among the New World clade lineages shown in Figure 4; relationships among members within each of these clades were not considered. For each hypothesis, we also examined support for both potential reconstructions of *B.* cf. *margaritifer*, although our interest in the placement of this species was secondary. These comparisons highlight the discrepancies between reconstructions with different optimality criteria. Only 20 trees in the Bayesian sample (bpp = 0.004) were consistent with the best parsimony tree (Fig. 4a; TL = 5008). Similarly, the most-parsimonious tree consistent with the maximum-likelihood topology (as depicted in Fig. 4b) has a much greater tree length than the best parsimony tree (difference = 12; Fig. 4b vs. Fig. 4a). This difference is largely due to the variable placement of *B.* cf. *margaritifer*. The more parsimonious topologies always reconstruct *B.* cf. *margaritifer* as outside of the New World clade (Fig. 4, comparisons of tree lengths between the first two columns). However, regardless of the placement of *B.* cf. *margaritifer*, the likelihood and Bayesian analyses support either the South American *B. marinus* clade (Fig. 4b, bpp = 41.0) or the Middle American clade (Fig. 4c; bpp = 44.6) as the sister taxon of the Nearctic *Bufo*. Only the latter reconstruction is consistent with Maxson's (1984) hypothesis.

Hypothesis Testing

A GTR + Γ + I model of evolution best described all reduced-taxon datasets used in hypothesis testing. For the full dataset and all reduced-taxon datasets, a molecular clock could not be enforced. Using parametric bootstrapping, the Nearctic polyphyly and Nearctic paraphyly hypotheses were each rejected ($P < 0.001$). Blair's (1972a) interpretation of two distinct clades representing the wide-skulled and narrow-skulled lineages is the basis of the Nearctic paraphyly hypothesis (Fig. 1b). However, because the monophyly of the wide-skulled group is not relevant to the biogeographic history of the North American and Nearctic *Bufo*, the wide-skulled taxa were not constrained to be monophyletic as part of the null for the Nearctic paraphyly hypothesis. To test Blair's (1972a,d) hypothesis of wide- versus narrow-skulled lineages, the wide-skulled South American and African taxa were constrained for monophyly as part of the null hypothesis for further testing (see Appendix 2, constraints 2 and 3). This hypothesis was also rejected ($P < 0.001$).

Additionally, we examined the hypothesis that the Nearctic *Bufo* are not monophyletic. To simulate data, we used the most-parsimonious tree (with the best likelihood score) that lacked Nearctic monophyly, which was found by searching for trees not compatible with the constraint of Nearctic monophyly. In this tree, the *B. boreas* group plus the *B. marinus* Clade are monophyletic, and this clade is the sister taxon of the remaining Nearctic *Bufo*. Nonmonophyly of the Nearctic *Bufo* was rejected ($P = 0.036$), further supporting a Nearctic clade. Our data also reject the hypothesis that the Eurasian *B. bufo* is nested within or is the sister taxon of the Nearctic *Bufo* ($P = 0.003$). Because the relationship of the Eurasian *B. viridis* to these taxa was not always explicit in these hypotheses, *B. viridis* was excluded from this test.

To test the Nearctic monophyly hypothesis, we used reconstructions with different sister taxa of the Nearctic clade as the null hypotheses (see Appendix 2, constraints 4 and 5). The Nearctic monophyly hypothesis requires that the North American *Bufo* are monophyletic, which means the sister taxon of the Nearctic *Bufo* has to be Middle American. Our data failed to reject monophyly of the North American *Bufo* ($P = 0.063$); therefore, some or all of the Middle American *Bufo* may be the sister lineage of the Nearctic *Bufo*. The Middle American *Bufo* were not constrained to be monophyletic in this test because the Nearctic monophyly hypothesis only predicts monophyly of the Nearctic *Bufo* and the North American *Bufo* (see Appendix 2); the Middle American *Bufo* may therefore be monophyletic or paraphyletic. Our data also failed to reject a sister-taxon relationship between the Nearctic clade and a clade including members of the Middle American and *B. marinus* clades ($P = 0.17$).

In summary, the Nearctic polyphyly and Nearctic paraphyly hypotheses, the existence of monophyletic wide- and narrow-skulled groups, the nonmonophyly of the Nearctic *Bufo*, and a putative Eurasian-Nearctic relationship between *B. bufo* and the *B. boreas* group are not supported by our data. Similarly, our data cannot differentiate (at $P = 0.05$) whether the Nearctic clade is the sister taxon of: (1) the *B. marinus* clade; (2) a clade including some or all of the Middle American taxa; or (3) a clade including the members of the Middle American and *B. marinus* clades.

Character Evolution

Changes in skull type occurred a minimum of 13 times, although the exact number of times that narrow or wide skulls evolved cannot be determined because of ambiguous character state reconstruction (Fig. 5). Similarly, neither the wide- nor narrow-skulled groups were recovered as monophyletic. Although the Nearctic clade is a large component of the narrow-skulled lineage, other members of the narrow-skulled group are more closely related to members of the wide-skulled group. The *B. marinus* and Middle American clades were found to include taxa previously assigned to both narrow- and wide-skulled groups.

DISCUSSION
Biogeographical Hypothesis Testing

Because biogeographic hypotheses make explicit predictions about the relationships among taxa, phylogenetic in-
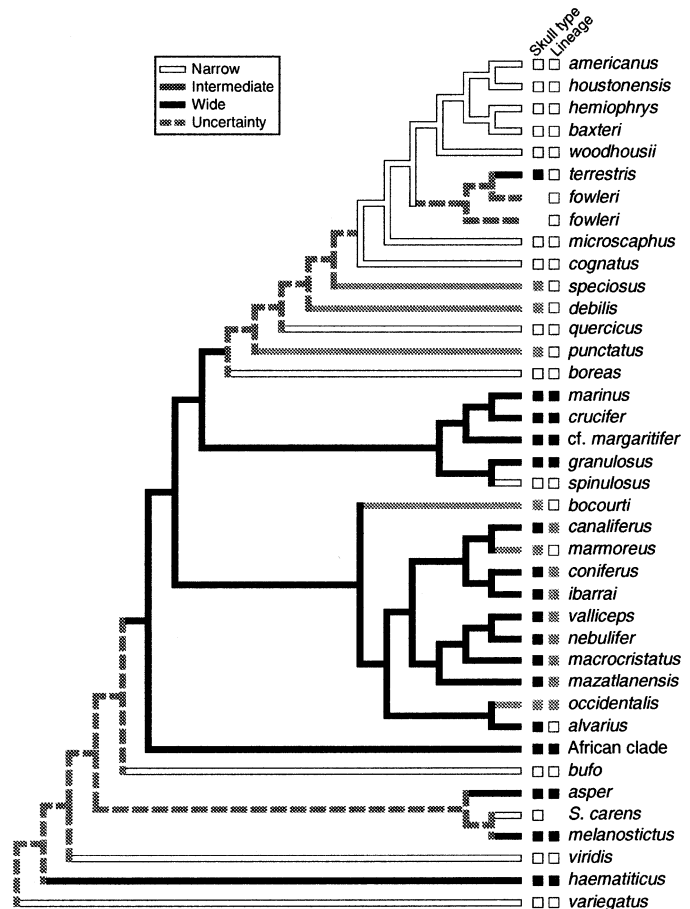

FIG. 5. Reconstruction of the evolution of frontoparietal/skull type on a condensed topology from the maximum likelihood analysis. The first column of squares at the tips of the branches is frontoparietal/skull type (following Martin 1972); the second column of squares represents the assignment of taxa to narrow-, intermediate-, and wide-skulled groups based on Blair (1972a,e). Narrow-skulled taxa are in white, intermediate-skulled taxa in gray, and wide-skulled taxa in black. Dashed gray lines indicate ambiguous state reconstructions. Absence of a square indicates insufficient information for assignment of character state.

vestigations are ideal for discriminating between competing hypotheses. Several methods exist for evaluating competing phylogenetic hypotheses, and their utility for addressing a variety of biological questions has been discussed previously (Huelsenbeck et al. 1996a,b; Huelsenbeck and Rannala 1997; Goldman et al. 2000; Buckley 2002). Although testing competing biogeographic hypotheses is a common motivation for many molecular phylogenetic studies, only a few studies have used statistical tests of explicit hypotheses (Steppan et al. 1999; Macey et al. 2000; Sullivan et al. 2000; Evans et al. 2003; Simpson et al., in press).

We used parametric bootstrapping to test biogeographic hypotheses regarding the Nearctic *Bufo*. Contrary to the Nearctic polyphyly hypothesis, our results suggest the Nearctic *Bufo* evolved as part of a northward radiation from a South American ancestor. Moreover, this radiation did not include intercontinental dispersal from the Nearctic into Eurasia as suggested by the Nearctic paraphyly hypothesis. Admittedly, our taxon sampling did not include any members of the Eur-

asian *B. calamita* and *B. stomaticus* species groups. We did include, however, *B. bufo* and *B. viridis*, which were the Eurasian lineages considered to be closely related to Nearctic *Bufo*, especially the *B. boreas* group (Tihen 1962a; Blair 1972a; Low 1972; R. F. Martin 1972; Goebel 1996).

These findings of Nearctic monophyly and a New World clade are consistent with Maxson's (1984) hypothesis for the origin of the Nearctic toads. The reconstruction of several South American lineages as basal to Eurasian and African taxa and the New World clade is also in accord with the Gondwanan origin that Maxson (1984) suggested (Fig. 2), although her limited sampling did not recover paraphyly of the South American *Bufo*. Maxson also argued that the Nearctic and Middle American *Bufo* evolved as a single, northward radiation from a South American ancestor. Our results are not definitive regarding the sister taxon of the Nearctic *Bufo*. The sister taxon may be the *B. marinus* clade (Fig. 4a,b) as preferred in the maximum likelihood and parsimony analyses; some or all of the Middle American taxa, as suggested by Maxson (1984; as in our Fig. 4c, although the Middle American taxa can also be paraphyletic which is not depicted); or a clade including all members of both of these two groups (Fig. 4d). Only Maxson's (1984) hypothesis suggests that a single invasion of North America was the only event leading to the North American *Bufo*. The other hypotheses require either two invasions of North America (as in Fig. 4b,d) or a single invasion with subsequent dispersal back to South America (as in Fig. 4a,b). Our data are unable to reject statistically any of these hypotheses. The Bayesian analysis, however, suggests the sister taxon of the Nearctic clade is either the *B. marinus* clade (Fig. 4b: 41.0%) or the Middle American clade (Fig 4c: 44.7%; bpp = 46 if *B. bocourti* is not constrained to be in the Middle American clade) as opposed to a clade containing both groups (Fig. 4d: 12.5%).

New World Bufo

Our finding of a Nearctic clade and a large New World clade conflict with most previous interpretations of *Bufo* relationships except for those of Maxson (1984). Nonmonophyly of the Nearctic *Bufo* has been suggested by several authors (Baldauf 1959; Sanders 1961; Tihen 1962a; Sanders and Cross 1964; Blair 1972d; Cardellini et al. 1984; Goebel 1996; Graybeal 1997), while only Maxson (1984) and Graybeal's (1997) combined morphological and molecular analysis have suggested monophyly of the Nearctic *Bufo*.

Why should the present results be accepted instead of previous interpretations? The studies conducted prior to Maxson's (1984; Maxson et al. 1981) investigations examined morphology, karyology/cytology, biogenic amines, parotoid gland secretions, blood proteins, vocalizations, and postzygotic genetic compatibility (Baldauf 1959; Sanders 1961; Sanders and Cross 1964; Blair 1972a; Bogart 1972; Cei et al. 1972; Guttman 1972; Low 1972; R. F. Martin 1972; W. F. Martin 1972; Szarski 1972). Because phylogenetic methodology at that time was not well developed, interpretation of these datasets was based on overall similarity. Moreover, the phylogenetic utility of a given data type was often difficult to interpret. For example, the extent to which protein simi-

larities in parotoid gland venom or genetic compatibility measured from hybridization studies was an accurate proxy of phylogenetic relatedness was, and remains, unknown (Porter and Porter 1967; Blair 1963, 1972c; Low 1972). Blair recognized these drawbacks and argued that each data type could only generate or support a tentative phylogeny, but that multiple lines of evidence taken together (e.g., Blair 1972d), might be able to elucidate the underlying phylogeny. Nevertheless, these conclusions were still hampered by the limitations of available phylogenetic methodology and the subjectivity of overall similarity so the conflict with other datasets is not surprising.

Conflict between the more recent mtDNA analyses could be attributable to differences in taxon sampling, data quality, and/or data quantity. Comparisons to Graybeal's (1997) results may be particularly impacted by low quality of the16S data resulting from changes in sequencing technology. Her 16S data were collected through automated sequencing, but her 12S data were obtained via manual sequencing. Graybeal (1997) noted that sequence divergences up to 1.1% were recovered when the same region in the same individual was sequenced using both methods. Resequencing of 16S from the same individuals as those used by Graybeal (1997) has yielded sequence divergences much greater than 1.1% (2.2% in Harris 2001; 1.8–10.2%, mean = 5.2%, *n* = 14, in our study). Graybeal's (1997) 12S data do not show this pattern. Goebel's (1996, fig. 12) mtDNA analysis, which included far fewer non-Nearctic *Bufo* than either Graybeal's (1997) study or our study, also suggested nonmonophyly of the Nearctic *Bufo*, but the relevant nodes were all very weakly supported.

Morphological Homoplasy

Both Tihen's (1962a) and Blair's (1972a) hypotheses were strongly influenced by osteological data. Osteological similarities between some Eurasian taxa, such as *B. bufo* and the *B. boreas* group, were a major factor in suggesting the non-monophyly of the Nearctic taxa. Moreover, the osteological categories, narrow- and wide-skulled, represent the two major *Bufo* lineages described by Blair (1972a,c,e). Although named for osteological characters, assignment to a lineage was based on a variety of data (see Blair 1972a,d). Therefore, it is possible to be osteologically narrow-skulled but to be placed in the wide-skulled lineage, as was suggested for *B. terrestris* and *B. alvarius*, and the opposite is true as well (Fig. 5).

The osteological definitions of wide- and narrow-skulled were based on frontoparietal characteristics. According to R. F. Martin (1972, 1973), intraspecific and intralineage variation in these characteristics was quite large, and he cautioned the use of osteological data in reconstructing bufonid relationships. Nevertheless, although Blair (1972a) suggested that narrow-skulled toads are cold adapted and tend to inhabit montane areas, he downplayed the possibility of convergence on frontoparietal type and regarded this suite of osteological and ecological characters as evidence for monophyly of the wide- and narrow-skulled groups. Martin (1973), however, suggested that convergence might result from repeated, independent reductions in skull weight, which may facilitate increased mobility and colonization of colder, upland envi-

ronments. Our results indicate extreme homoplasy in frontoparietal type, and our dataset does not recover monophyletic wide- and narrow-skulled lineages (Fig. 5). Graybeal (1997) also concluded that there was little evidence for the monophyly of these groups, although the topological results of our study and hers that lead to this conclusion do differ.

The Nearctic-Neotropical Boundary

The major biogeographic regions were demarcated based upon congruent distributional patterns. Regional boundaries, therefore, are transition zones or areas of limited dispersal. Taxa such as *Bufo* whose distributions span these zones may seem to argue against the interpretation of regional boundaries as barriers to dispersal. Here, however, we have demonstrated a single colonization event for the origin of the Nearctic *Bufo*. This suggests that even in this widespread group, historical dispersal across the Nearctic-Neotropical boundary was rare.

The Nearctic-Neotropical boundary runs from the Rio Grande Valley around the central highlands of Mexico, including the Central Plateau and the Sierra Madre Oriental and Occidental, to the central Sinaloan Coast of Mexico. The distributions of *Bufo* species suggest that this boundary is a broad, transition zone rather than a narrow, easily demarcated boundary. The southern distributional limits of several Nearctic species including *B. cognatus*, *B. mexicanus*, *B. retiformis*, *B. kelloggi*, and *B. compactilis* coincide with this boundary. Similarly, the northern limit of the range of the marine toad, *B. marinus*, abuts this boundary along the Gulf of Mexico. However, other *Bufo* have distributions that cross the boundary. *Bufo speciosus*, *B. punctatus*, and *B. debilis* extend into the coastal areas of the northern Neotropical region (Stebbins 1985), and *B. nebulifer*, *B. mazatlanensis*, and *B. alvarius* range into the southern Nearctic (Conant and Collins [1998], Porter [1963], and Stebbins [1985], respectively).

Wallace (1876, Vol. 1, p. 58) also noted the ''composite character'' of the Mexican fauna and argued that a single distinct line does not accurately represent the variation in distributions of different taxa (Vol. 2, p. 117). For example, of the 13 nonendemic amphibian families in Middle America, eight reach either their northern or southern limits in this region (Campbell 1999). Interestingly, the limits of five of these families coincide with the southeastern extent of the Nearctic region near the Isthmus of Tehuantepec (Campbell 1999, fig. 3.3). Studies of the distributions of New World bats (Ortega and Arita 1998), mammals (Brown and Lomolino 1998, fig. 10.11), and freshwater fish (Miller 1966) have found similar patterns of a broad transition zone between the Nearctic and Neotropical regions, and Halffter (1987) described this region as the Mexican Transitional Zone based on studies of the insect fauna. The lack of a single distinct biogeographic barrier at the delineated boundary is also demonstrated by the Mexican Neovolcanic Plateau, which is approximately 700 km south of the boundary but acts as an important barrier along the Gulf Coastal Plain in toads, mammals, reptiles, and fish (Pérez-Higaredera and Navarro 1980; Miller 1986; Mulcahy and Mendelson 2000; Hulsey et al. 2004). Therefore, although the Nearctic-Neotropical boundary is an important barrier that has impacted the phylogenetic

relationships of *Bufo* and distributional patterns of many organisms, this boundary, like that between the Australian and Oriental faunas (e.g., Simpson 1977; Evans et al. 2003), is not a narrow line but a broader transition zone.

Timing and Routes of Nearctic Colonization

Maxson (1984) argued that the diversification of *Bufo* resulted from Gondwanan vicariance followed by invasions from South America into North America and from Africa into Eurasia. Although she did not suggest when *Bufo* might have entered North America or the Nearctic, Savage (1966, 1973) suggested that *Bufo* and several anuran groups dispersed across the Isthmian Link into tropical North America in the Paleocene (58–65 million years ago). The Isthmian Link is hypothesized to have formed following a Late Cretaceous drop in sea level that resulted in a Paleocene land connection between the Nearctic and South America; this connection subsided by the Eocene. Evidence for this land bridge includes dispersal patterns of several terrestrial species and vicariance patterns of a few marine taxa (Gayet et al. 1992; Briggs 1994). Geophysical data, however, do not support a contiguous Paleocene land bridge (Duque-Caro 1990; Pitman et al. 1993). Sea-level estimates are also not consistent with the Isthmian Link. The drop in sea level between 66 and 68 million years ago was of short duration, and the Paleocene was marked by higher sea levels before another drop at the Paleocene-Eocene boundary (Haq et al. 1987). However, geophysical data and paleogeographic models suggest that island-hopping dispersal across the Antilles and Aves Ridge from the late Cretaceous to the mid-Eocene (about 49 million years ago) may have permitted movement between North and South America for some terrestrial species (Pitman et al. 1993). More recently, dispersal across the developing Central American Land Bridge, which exists today, may have been possible for some taxa as early as the mid-Miocene (Duque-Caro 1990; Pitman et al. 1993).

To examine the timing and potential routes of colonization, the fossil record and phylogenetically based age estimates can be used. Maxson's (1984) hypothesis predicts that the earliest *Bufo* fossils should be on western Gondwanan landmasses and be older than the timing of separation of these land masses (about 100 million years ago). Similarly, Savage's (1973) hypothesis of Paleocene dispersal would be supported by the occurrence of Cretaceous *Bufo* fossils in South America. The earliest reported *Bufo* species are from the Paleocene of South America and the Oligocene (Whitneyan; about 29 million years ago) of Florida, but these are undescribed (Báez and Gasparini 1979; Patton 1969). The first well-documented *Bufo* fossil is *B. praevius* from the late lower Miocene (20–23.3 million years ago) of Florida (Tihen 1951, 1962b), but it is only identified with certainty to genus (Tihen 1972). Miocene *Bufo* are also found in Eurasia, Africa, and South America suggesting a pre-Miocene origin for *Bufo* (Tihen 1972). Therefore, at present, there are no Cretaceous fossils to support Gondwanan origin and/or Paleocene dispersal hypotheses. Additionally, if *B. praevius* is found to be part of the Nearctic clade, then dispersal across the Central American Land Bridge since the mid-Miocene can be ruled out as the original colonization route.

These hypotheses also predict minimum times of divergence for particular nodes. To estimate divergence times, the minimum age of at least one clade must be determined from fossil evidence. Unfortunately, the *Bufo* fossil record is depauperate (Tihen 1972; Sanchíz 1998); most described fossils are identified as extant forms (Sanchíz 1998), based largely on the ilium (G. S. Bever and C. J. Bell, pers. comm.), and, at least for North American *Bufo*, polymorphism and overlap of quantitative ilial characters precludes phylogenetic assessment (Bever 2002). As a result, the use of fossils for age calibration is not currently possible.

Fossil and biogeographic data suggest that colonization of North America by *Bufo* occurred prior to the development of a contiguous Central American Land Bridge (3.1–3.7 million years ago; Duque-Caro 1990). Nearctic *Bufo* fossils are older than the formation of a complete land bridge (Sanchíz 1998). Additionally, the estimated divergence time of the Middle American *B. valliceps* and *B. nebulifer* at the Mexican Neovolcanic Plateau is 4.2–7.6 million years ago (Mulcahy and Mendelson 2000). This suggests that Middle American *Bufo* were in central Mexico before 3.7 million years ago.

Transmarine dispersal presents a paradox because with their permeable skins and low salt-water tolerance, amphibians should be poor dispersers across marine barriers. Nevertheless, several recent studies have demonstrated transmarine dispersal. In Southeast Asia, studies of the *Rana signata* complex (Brown and Guttman 2002) and fanged frogs (*Limnonectes*; Evans et al. 2003) have demonstrated multiple crossings of marine barriers, including Wallace's Line. Endemic anurans on the oceanic island Mayotte and hyperoliid frogs on Madagascar and the Seychelles have also undergone transmarine dispersal (Vences et al. 2003).

Transmarine dispersal between North and South America has also been implicated in other taxa (Marshall et al. 1983; Gayet et al. 1992; Engel et al. 1998). Fossil data suggest that three mammalian taxa (edentates, notoungulates, and dinocerates) in the late Paleocene (Gingerich 1985) and the hylid and microhylid frogs in the Oligocene (Estes and Báez 1985) colonized the Nearctic from the Neotropics. In plants, phylogenetic studies of *Hoffmannseggia* and Malpighiaceae suggest multiple nonsimultaneous colonizations of the Nearctic via long-distance dispersal (Simpson et al. [in press] and Davis et al. [2002], respectively). However, long-distance or island-hopping dispersal across marine barriers or other inhospitable habitats should be more common in many plants than in nonvolant vertebrates. In the New World, sigmodontine rodents colonized South America from the Nearctic in the Late Miocene (Engel et al. 1998), but we lack phylogenetic studies of nonvolant, New World vertebrates with distributions amenable to testing hypotheses of Nearctic colonization by Neotropical ancestors prior to the formation of a complete land bridge and the ensuing Great American Interchange. Nearctic colonization by Palearctic ancestors has been demonstrated in snake and mammalian taxa (Kraus et al. 1996; Parkinson 1999; Conroy and Cook 2000; Stone and Cook 2002). Among amphibians, colonization of the Nearctic from the Neotropics prior to a complete land bridge presumably occurred in some frogs (hylids, microhylids, and leptodactylids; Estes and Báez 1985; Vanzolini and Heyer 1985), but has not been demonstrated in a hypothesis-driven context.

This study of *Bufo* is the first to test explicitly for colonization from the Neotropics prior to the Great American Interchange by nonvolant vertebrates. Future studies of other nonvolant vertebrates will add to the emerging role of the Central American Land Bridge as a possible colonization route and our understanding of the development of the Nearctic biota.

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APPENDIX 1

Specimens sampled, locality data, and GenBank accession numbers. Assignment of species to species group and geographical region is also noted. Numbers in parentheses following taxon names correspond to Appendix 2; if two conspecific individuals were collected in the same state, unique identifiers are also listed in parentheses. Voucher numbers in parentheses are field identification numbers for which museum numbers were not available.

Taxa	Species group	Voucher number	Locality	GenBank number
Nearctic *Bufo*				
Bufo americanus americanus (1)	americanus	MVZ 223282	Lousiana: East Feliciana Parish	AY680204
Bufo americanus americanus (2)	americanus	MVZ 143049	Ohio: Lorain: 8 km N of Oberlin	AY680211
Bufo americanus charlesmithi (3)	americanus	TNHC 62701	Kansas: Cherokee: along Spring River, at Riverton	AY680206
Bufo americanus charlesmithi (4)	americanus	TNHC (DMH 88–79)	Oklahoma: Leflore: 1.6 km S of Bohoshe	AY680205
Bufo baxteri (5)	americanus	TNHC 62699	Wyoming: Albany: USFWS captive breeding program at Sybille Wildlife Research Center from wild stock at Mortenson Lake National Wildlife Refuge	AY680207
Bufo californicus (6)	americanus	CAS 175636	California: San Diego: Cleveland National Forest: Kitchen Creek: 1.1 km S of Cibbets Flat Campground	AY680225
Bufo fowleri (7)	americanus	TNHC 61997	New York: Suffolk: Brookhaven Township: Gordon Heights: 0.8 km NE of intersection of Granny Rd. and SR112	AY680212
Bufo fowleri (8; NC-1)	americanus	MVZ 223384	North Carolina: Chatham: Wiseacre Farm: ~3.2 km from Pittsboro on Alston Chapel Rd.	AY680224
Bufo fowleri (9; NC-2)	americanus	TNHC 61996	North Carolina: Stanley: ~2.4 km E of Hwy 125 on SR1741	AY680223
Bufo hemiophrys (10)	americanus	MVZ 137738	Minnesota: Traverse: vicinity of Wheaton	AY680213
Bufo houstonensis (11)	americanus	TNHC (DMH 88-3)	Texas: Bastrop: Big Fence Pond, along Route 21	AY680208
Bufo microscaphus (12)	americanus	MVZ 223365	Arizona: Yavapai: Vicinity of Dewey	AY680227
Bufo microscaphus (13; NM-1)	americanus	USNM 311140	New Mexico: Catron: Bull Pass Tank, 8 km N and 57 km W of Winston	AY325989
Bufo microscaphus (14; NM-2)	americanus	USNM 311161	New Mexico: Socorro: Alamosa Warm Springs	AY680226
Bufo terrestris (15)	americanus	CAS 207171	Alabama: Escambia: Conecuh National Forest: Hwy 11, 0.4 km S of Hwy 4	AY680222
Bufo terrestris (16)	americanus	MVZ 223379	Florida: Charlotte: vicinity of Punta Gorda	AY680220
Bufo terrestris (17)	americanus	TNHC 61995	South Carolina: Jasper: B&C Landing Rd.	AY680221
Bufo velatus (18; TX-1)	americanus	TNHC 61057	Texas: Jasper: road along Big Sandy Creek, 5.8 km S of jct. of Hwy 190 and FM 777	AY680209
Bufo velatus (19; TX-2)	americanus	TNHC 55593	Texas: Marion: Smithland: 4.8 km N and 5.1 km W of jct. of Hwys 43 and 49	AY680210
Bufo woodhousii australis (20; AZ-1)	americanus	UTA 53926	Arizona: Pinal: Dudleyville	AY680216
Bufo woodhousii (21)	americanus	TNHC 60510	New Mexico: Socorro: Veguita	AY680219
Bufo woodhousii woodhousii (22; AZ-2)	americanus	UTA 56499	Arizona: Yuma: County 14th St., ~1.6 km E of Somerton Ave.	AY680218
Bufo woodhousii woodhousii (23)	americanus	MVZ 137737	Nevada: Clark: Overton	AY680215
Bufo woodhousii woodhousii (24)	americanus	TNHC 60511	Texas: King: on FM 193, 1.7 km E of Dickens/King Co. line	AY680217
Bufo woodhousii woodhousii (25)	americanus	MVZ 226107	Washington: Benton: Whitcomb Island in Columbia River	AY680214
Bufo boreas boreas (26)	boreas	MVZ 225512	Alaska: Annette Island: Metlakatla Peninsula: vicinity of Annette Island Airport	AY680241
Bufo boreas boreas (27)	boreas	MVZ 142827	Oregon: Lake: Warner Mtns: US Forest Service Rd. 3615, Mud Creek at SE edge of Bull Prairie	AY680242
Bufo boreas halophilus (28)	boreas	MVZ 145277	California: San Diego: 11.2 km SW of Warner Spring at jct. of Hwys 76 and 79	AY680244
Bufo canorus (29; CA-1)	boreas	MVZ 142987	California: Mono: E of Sonora Pass	AY680238
Bufo canorus (30; CA-2)	boreas	MVZ 142992	California: Mono: Lake Mary	AY680239
Bufo exsul (31)	boreas	MVZ 137717	California: Inyo: Deep Springs Valley: Buckhorn Spring	AY680243
Bufo nelsoni (32)	boreas	MVZ 142829	Nevada: Nye: 11.9 km W of Beatty on Hwy 95	AY680240
Bufo cognatus (33)	cognatus	MVZ 143007	Arizona: Cochise: Coronado National Forest, along Road 42	AY680231
Bufo cognatus (34)	cognatus	MVZ 143048	Minnesota: Traverse: vicinity of Wheaton	AY680230

APPENDIX 1
Continued.

Taxa	Species group	Voucher number	Locality	GenBank number
Bufo speciosus (35; TX-1)	cognatus	MVZ 143046	Texas: Brewster: 65.1 km E of Marathon on Hwy 90	AY680228
Bufo speciosus (36; TX-2)	cognatus	TNHC 60379	Texas: Cottle: on road 3256, just E of Matador Wildlife Management Area	AY680229
Bufo debilis debilis (37)	debilis	TNHC (AG 137)	Texas: Wise: Lyndon B. Johnson National Grassland	AY680232
Bufo debilis insidior (38)	debilis	MVZ 223354	Arizona: Cochise: 8.6 km E of Portal on Portal Rd	AY680233
Bufo retiformis (39)	debilis	MVZ 222504	Arizona: Pima: 19.2 km N of Quijota on Indian Route 15	AY325982
Bufo punctatus (40)	punctatus	MVZ 142927	Mexico: Baja California Sur: dam, 3.2 km W of Agua Caliente	AY680237
Bufo punctatus (41)	punctatus	TNHC 58788	Texas: Gillespie: 3.8 km N of Willow City on Willow City Loop Rd.	AY680236
Bufo quercicus (42)	quercicus	MVZ 223370	Florida: Charlotte: N of Cleveland at jct. of Hwy 17 and West Washington Loop Rd.	AY680235
Bufo quercicus (43)	quercicus	TNHC 61998	South Carolina: Berkeley: Francis Marion National Forest, 1.1 km from county line on Hwy 200	—
Middle American *Bufo*				
Bufo alvarius (44)	alvarius	TNHC 61247	Arizona: Pima: N of Tucson	AY325984
Bufo bocourti (45)	bocourti	MVZ 143367	Guatemala: Huehuetenango: ~2 km NW of Barillas	AY680245
Bufo bocourti (46)	bocourti	UTA 13003	Mexico: Chiapas: Grutas de San Cristobal: 6.4 km SE of San Cristobal de las Casas	AY680246
Bufo canaliferus (47)	canaliferus	UTA 34110	Mexico: Oaxaca: 21 km E of Puerto Escondido	AY680251
Bufo coniferus (48)	coniferus	MVZ 203771	Costa Rica: Cartago: 1.4 km S of Tapantí Bridge on road to Refugio Nacional Tapanti	AY680247
Bufo fastidiosus (49)	fastidiosus	MVZ 217439	Costa Rica: Puntarenas: along Rio Coton, ~2 km SE of Las Tablas	AY680248
Bufo ibarrai (50)	coccifer	UTA 17117	Guatemala: Baja Verapaz: 3.2 km WNW of Purulhá	AY680249
Bufo marmoreus (51)	marmoreus	UTA 13032	Mexico: Oaxaca: 1.1 km NE of Tapanatepec	AY680250
Bufo occidentalis (52)	occidentalis	UTA 34111	Mexico: Oaxaca: 25.0 km N of San Gabriel Mixtepec	AY680257
Bufo tacanensis (53)	tacanensis	MVZ 170329	Mexico: Chiapas: Volcán Tacana: Colonia Talquian: 3 km N of Union Juarez	AY680258
Bufo macrocristatus (54)	valliceps	UTA 13014	Mexico: Chiapas: 16.1 km NW of Pueblo Nuevo Solistahuacan	AY680256
Bufo mazatlanensis (55)	valliceps	MVZ 132973	Mexico: Sonora: vicinity of Alamos	AY680254
Bufo melanochlorus (56)	valliceps	MVZ 229635	Costa Rica: Heredia Province: La Selva Biological Station	AY680255
Bufo nebulifer (57)	valliceps	UTA 13119	Mexico: Hidalgo: 38.5 km SW of Huejutla	AY680252
Bufo nebulifer (58)	valliceps	TNHC 62000	Texas: San Saba: Colorado Bend State Park	AY325985
Bufo valliceps (59)	valliceps	UTA 13097	Mexico: Chiapas: 1.6 km N of Chiapa del Corzo	AY680253
South American *Bufo*				
Bufo crucifer (60)	crucifer	ZUEC (DCC 3392)	Brazil: Rio de Janeiro: Magé, Campo de Escouteiras, Santo Aleixo	AY680260
Bufo granulosus (61)		USNM 302451	Brazil: Roraima: Caracarann	AY680261
Bufo haematiticus (62)	guttatus	MVZ 223359	Costa Rica: Heredia Province: ~ 14 km SE of San Isidro del General: at jct. of Rio Penas Blancas and Rio General	AY680270
Bufo cf. *margaritifer* (63)	margaritifer	ZUEC (DCC 3393)	Brazil: Rio de Janeiro: Magé, Campo de Escouteiras, Santo Aleixo	AY680262
Bufo marinus (64)	marinus	KU 202274	Ecuador: Pichincha: Tinalandia: 15.5 km SE of Santo Domingo de los Colorados	AY680259
Bufo marinus (65)	marinus	KU 205236	Peru: Madre de Dios: Cusco Amazónico	AY325994
Bufo spinulosus (66)	spinulosus	(NB 96-23)	Argentina: Neuquén: Laguna Blanca	AY680263
Bufo variegatus (67)	variegatus	(NB 9-19)	Argentina: Lago del Desierto	AY680269

APPENDIX 1
Continued.

Taxa	Species group	Voucher number	Locality	GenBank number
Eurasian *Bufo*				
Bufo asper (68)	asper	TNHC 53891	pet trade (Java)	AY680266
Bufo biporcatus (69)	biporcatus	TNHC 61079	pet trade (SE Asia)	AY325987
Bufo bufo (70)	bufo	TNHC 56744	USSR: Latvian Republic: Riga	AY325988
Bufo viridis (71)	viridis	TNHC 56752	USSR: Ukranian Republic: Kiev Region	AY680267
Bufo melanostictus (72)	melanostictus	TNHC 59161	Indonesia: West Java Province: Depok: University of Indonesia Campus	AY680268
African *Bufo*				
Bufo mauritanicus (73)	mauritanicus	MVZ 164714	Morocco: Errachidia Province: Tafilalt Oasis: Erfoud	AY680265
Bufo kisoloensis (74)	regularis	MVZ 223361	Uganda: Rukungiri District: Bwindi Forest Reserve: Buhoma	AY325995
Bufo regularis (75)	regularis	TNHC 61999	pet trade (N Africa)	AY680264
Bufo steindachneri (76)	steindachneri	MVZ 223373	Kenya: Arubuko Sokoka Forest	AY325981
Non-*Bufo* bufonids				
Osornophryne guacamayo (77)		QCAZ 4580	Ecuador: Napo Province: Volcán Sumaco: Lago Sumaco	AY326036
Schismaderma carens (78)		TNHC 62001	pet trade (from Dodoma, Tanzania)	AY325997
Outgroups				
Ceratophrys cornuta (79)		KU 205076	Peru: Madre de Dios: Cusco Amazónico	AY326014
Eleutherodactylus w-nigrum (80)		KU 202561	Ecuador: Carchi: ~5 km W of La Gruel	AY326004
Hyla cinerea (81)		TNHC 61054	Texas: Jasper: road along Big Sandy Creek, 5.8 km S of jct. of Hwy 190 and FM 777	AY680271
Physalaemus gracilis (82)		(AJC 95-228)	Brazil: Porto Alegre: Rio Grande do Sul	AY680272

Constraint trees used in parametric bootstrapping. Constraint trees for some of the parametric bootstraps are not provided here because they are described in the Results section. Numbers correspond to the taxa listed in Appendix 1. For all parametric bootstraps, taxa 14 and 29 were excluded because they are identical to other taxa in the analysis (see Materials and Methods). Some species in the *Bufo americanus* group were represented by several closely related sequenes so eight additional taxa were excluded to reduce computational time. These are 3, 4, 15, 18, 19, 22, 23, and 25. Except for *B*. cf. *margaritifer* in the fifth constraint listed, taxa not listed in the parenthetical notation were excluded from the parametric bootstrap because insufficient information was available to allow for placement in the constraint tree.

(1) Constraint tree representing the Nearctic polyphyly hypothesis. ((((26, 27, 28, 30, 31, 32), 70), (1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 20, 21, 24, 33, 34, 35, 36), (37, 38, 39, 40, 41, 42, 43, 61, 63), (60, (64, 65)), (44, 47, 48, 51, 52, 54, 55, 57, 58, 59)), 62, 66, 73, 74, 75, 76, 77, 79, 80, 81, 82)

(2) Constraint tree representing the Nearctic paraphyly hypothesis. (((((1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 20, 21, 24, 26, 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 66, (68, 71)), (49, 54, 55, 56, 57, 58, 59)), 47, 52), 60, 64, 65, 72, 73, 74, 75, 76), 77, 79, 80, 81, 82)

(3) Constraint tree representing Blair's (1972a,d) hypothesis of wide- and narrow-skulled groups (same as Nearctic paraphyly hypothesis except the wide-skulled taxa are constrained as monophyletic. (((((1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 20, 21, 24, 26, 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 66, (68, 71)), (49, 54, 55, 56, 57, 58, 59)), 47, 52), (60, 64, 65, 72, 73, 74, 75, 76)), 77, 79, 80, 81, 82)

(4) Constraint tree for testing monophyly of the North American taxa. This tree was used to find the most parsimonious trees not compatible with the constraint. (((1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 20, 21, 24, 26, 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43), 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59), 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82)

(5) Constraint tree for testing if the sister lineage to the Nearctic *Bufo* could be a clade including some or all of the Middle American and *B. marinus* group members. A backbone constraint was used so that the reconstruction of *B*. cf. *margaritifer* was not constrained. Identical to the constraint for North American monophyly except *B*. cf. *margaritifer* is excluded and North America and the *B. marinus* clade are not constrained to be monophyletic. (((1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 20, 21, 24, 26, 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43), (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59)), 60, 61, 62, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82)