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Phylogenetic Systematics, Historical Biogeography, and the Evolution of Vocalizations in Nearctic Toads (*Bufo*)

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Phylogenetic Systematics, Historical Biogeography, and the Evolution of Vocalizations in Nearctic Toads (*Bufo*)

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Dedication

To my parents, Matthew and Georgia, for many years of support and encouragement.

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Phylogenetic Systematics, Historical Biogeography, and the Evolution of Vocalizations in Nearctic Toads (*Bufo*)

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The evolution of mating signals has long interested biologists because changes in mating signal production and/or reception can lead to reproductive isolation and speciation. Here, I examine the evolution of the male mating signal (the advertisement call) and the female preference for this call in the Western Toad, *Bufo boreas*. Call surveys and a morphological study for the occurrence of vocal sacs, which are necessary for producing these calls, reveal that only populations in the northeastern corner of this species' range produce long, high-amplitude advertisement calls. This is the first study to report among-population variation in the presence of the major mating signal in any animal. Although populations vary in whether or not males call, phonotaxis tests demonstrate that female *B. boreas* in calling and non-calling populations have the preference for this call. Phylogenetic analyses indicate that the call was lost in the

ancestor to modern *B. boreas* and then secondarily re-evolved in the northeastern populations.

Bufo boreas is one of many toad species that inhabits the Nearctic region. I use phylogenetic analyses of large and small subunit mitochondrial ribosomal DNA sequences to examine the phylogenetic relationships among Nearctic toad species and test previously proposed biogeographic hypotheses for the colonization history of the Nearctic region. This work indicates that the Nearctic *Bufo* are monophyletic and result from a single colonization event from the Neotropics. Further, fossil and paleogeographic data suggest that this colonization occurred prior to the formation of a contiguous land bridge between the Neotropic and Nearctic regions.

Many of the individuals examined in the Nearctic toad study had previously been sequenced for the same gene region. A surprising number of errors were found in the earlier sequences and attributed to the method of sequence generation. In my final chapter, I review the causes and consequences of sequencing error and present a novel method that uses sequence conservation information to detect errors. This approach is exemplified with the unique dataset of replicated sequences, and resources for easily implementing this approach are made available on the Comparative RNA Web Site (http://www.rna.ccbb.utexas.edu/).

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Chapter 1: The History of a Nearctic Colonization: Molecular Phylogenetics and Biogeography of the Nearctic Toads (*Bufo*)*

1.1 Introduction

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

*Significant portions of this chapter have been previously published as Pauly, Hillis, and Cannatella, 2004. Evolution 58: 2517–2535.

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 nearly cosmopolitan, 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 inter-continental 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. 1.1a). 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 of a narrow-skulled lineage that included some South American, Middle American, and Eurasian taxa (Fig. 1.1b). 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 morphological, 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. 1.1c). Additionally, in contrast to Blair (1972a), she 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 non-monophyly 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 (ca. 58 – 65 mya) 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 utilize 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 utilize 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*.

1.2 METHODS

The characterization of a taxon as either Nearctic, Middle American, or South American (Appendix A) 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 A). 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 A). 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.). Overlapping sets of primers were used to amplify approximately 2.5kb of the mitochondrial 12S and 16S genes and the intervening valine tRNA (Table 1.1). 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 PCR conditions (Palumbi, 1996) with the following thermal cycle profile: 2 min at 94 °C, followed by 35 cycles of: (94°C for 30 s, 46°C for 30 s, and 72°C for 60 s), 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 (Versions 2 and 3; Applied Biosystems), purified with Sephadex G-50 (Sigma #S-6022) in Centrisep columns (Princeton Separations #CS-901), and analyzed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Editing and assembly of contigs was completed using Sequencher 4.1 (Gene Codes Corp.).

Alignment and Phylogenetic Analyses

Initial alignment of DNA sequences was completed in Clustal X (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* (Version 4.0b10; Swofford, 2003). 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 non-optimal 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. 1.3). 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 utilized 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 B). Constraint trees are either described in the Results or provided in Appendix B. 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.

1.3 Results

Sequence Variation and Alignment

The DNA sequences are deposited in GenBank (Appendix A), 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 non-bufonid hyloids (Darst and Cannatella, 2004; DCC and DMH, 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 0.27–0.74 subs/site (0.27–0.48 subs/site with *Eleutherodactylus w-nigrum* excluded) and ranged up to 0.24 subs/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 (DCC and DMH, 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 utilized 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 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 subs/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 between 2 and 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 3 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 Fig. 1.2.

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. 1.2 and 1.3). 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. 1.2 and 1.4), 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. 1.3). 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. 1.2 and 1.3). The African bufonid *Schismaderma carens* is nested within *Bufo,* a result consistent with several previous studies (Graybeal, 1997; Maxson, 1981). 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. 1.2 and 1.3). 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.

Non-traditional 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, and R. F. Martin, 1972; but see Duellman and Schulte, 1992 and 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 other groups (Appendix A). 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 subs/site as compared to only 0.171 subs/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 subs/site. If *B.* cf. *margaritifer* is pruned from the population of trees used to

generate the Bayesian consensus tree, then high support for the monophyly of the New World Clade (bpp = 99) and the monophyly of the *B. marinus* Clade (bpp = 100) results (Fig. 1.2).

Middle American Clade.—In the likelihood analysis, the Middle American *Bufo* are monophyletic (bpp = 99), and *Bufo bocourti* is the sister taxon to the other Middle American taxa (Fig. 1.2). 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*; 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 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. 1.4a,b), although the Bayesian analysis shows the greatest support for (Nearctic Clade + Middle American Clade) (Fig. 1.4c), which is consistent with Maxson's (1984) hypothesis. The third arrangement, (Nearctic Clade (Middle American Clade + the *B. marinus* Clade)) (Fig. 1.4d), 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 Fig. 1.4; 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. 1.4a; TL = 5008). Similarly, the most-parsimonious tree consistent with the maximum-likelihood topology (as depicted in Fig. 1.4b) has a much greater tree length than the best parsimony tree (difference = 12; Fig. 1.4b vs. Fig 1.4a). 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. 1.4, 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. 1.4b, bpp = 41.0) or the Middle American clade (Fig. 1.4c; 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. 1.1b). 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 B, 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*. Non-monophyly 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 B, 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 B); 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 non-monophyly 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 thirteen times, although the exact number of times that narrow or wide skulls evolved cannot be determined because of ambiguous character state reconstruction (Fig. 1.5). 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.

1.4 DISCUSSION

Biogeographical Hypothesis Testing

Because biogeographic hypotheses make explicit predictions about the relationships among taxa, phylogenetic investigations 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; Sullivan et al., 2000; Macey et al., 2000; Evans et al., 2003; Simpson et al., 2005).

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 Eurasian *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. 1.2), although her limited sampling did not recover paraphyly of the South American *Bufo*. She 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. 1.4a,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. 1.4c, 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. 1.4d). 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. 1.4b,d) or a single invasion with subsequent dispersal back to South America (as in Fig. 1.4a,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. 1.4b: 41.0%) or the Middle American Clade (Fig 1.4c: 44.6%; bpp = 46 if *B. bocourti* is not constrained to be in the Middle American Clade) as opposed to a clade containing both groups (Fig. 1.4d: 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). Non-monophyly 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 post-zygotic 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 similarities in parotoid gland venom or genetic compatibility measured from hybridization studies was an accurate proxy of phylogenetic relatedness was, and remains, unknown (Blair, 1963, 1972c; Low, 1972; Porter and Porter, 1967). 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%, avg. = 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 non-monophyly 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. 1.5).

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 environments. Our results indicate extreme homoplasy in frontoparietal type, and our dataset does not recover monophyletic wide- and narrow-skulled lineages (Fig. 1.5). 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) also noted the "composite character (Vol. 1, p. 58)" 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 non-endemic 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 (Miller, 1986; Mulcahy and Mendelson, 2000; Pérez-Higaredera and Navarro, 1980; 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., Evans et al., 2003; Simpson, 1977), 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 mya). 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 (Briggs, 1994; Gayet et al., 1992). 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 mya 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 (ca. 49 mya) 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 (approximately 100 mya). 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 sp.* are from the Paleocene of South America and the Oligocene (Whitneyan; ~29mya) of Florida, but these are undescribed (Baez and Gasparini, 1979; Patton, 1969). The first well-documented *Bufo* fossil is *B. praevius* from the late lower Miocene (20–23.3 mya) 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 (Sanchíz, 1998; Tihen, 1972); most described fossils are identified as extant forms (Sanchíz, 1998), based largely on the ilium (Bever, 2005), and, at least for North American *Bufo*, polymorphism and overlap of quantitative ilial characters precludes phylogenetic assessment (Bever, 2005). 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 mya; 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 mya (Mulcahy and Mendelson, 2000). This suggests that Middle American *Bufo* were in central Mexico before 3.7 mya.

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 (Gayet et al., 1992; Marshall et al., 1983; 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 *Hoffmanseggia* and Malpighiaceae suggest multiple non-simultaneous colonizations of the Nearctic via long-distance dispersal (Simpson et al., 2005 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 non-volant 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 non-volant, 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 an 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 non-volant vertebrates. Future studies of other non-volant 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.

TABLE 1.1. 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 DMH and DCC, including modified versions of primers listed in Goebel et al. (1999).

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

[a]Primary primers used.
[b]Secondary primers used for only a fraction of the individuals.
[c]Used only for *B. baxteri*.

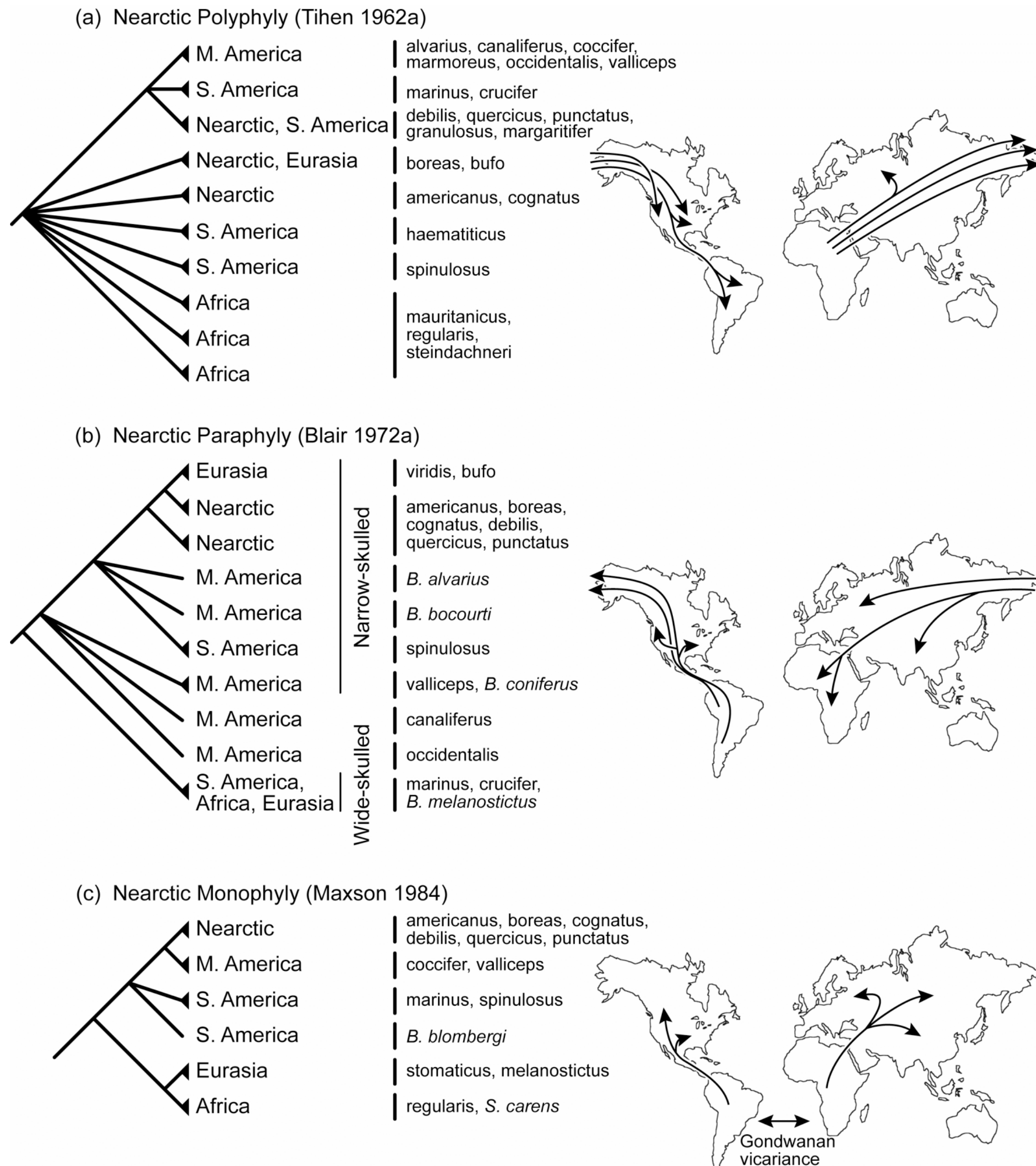


FIGURE 1.1. Tree topologies and potential dispersal/colonization routes representative of previously proposed biogeographic hypotheses for the origin of the Nearctic *Bufo.* Species groups and species (italicized) listed to the right of each topology represent the taxa sampled in each study. (a) Nearctic 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).

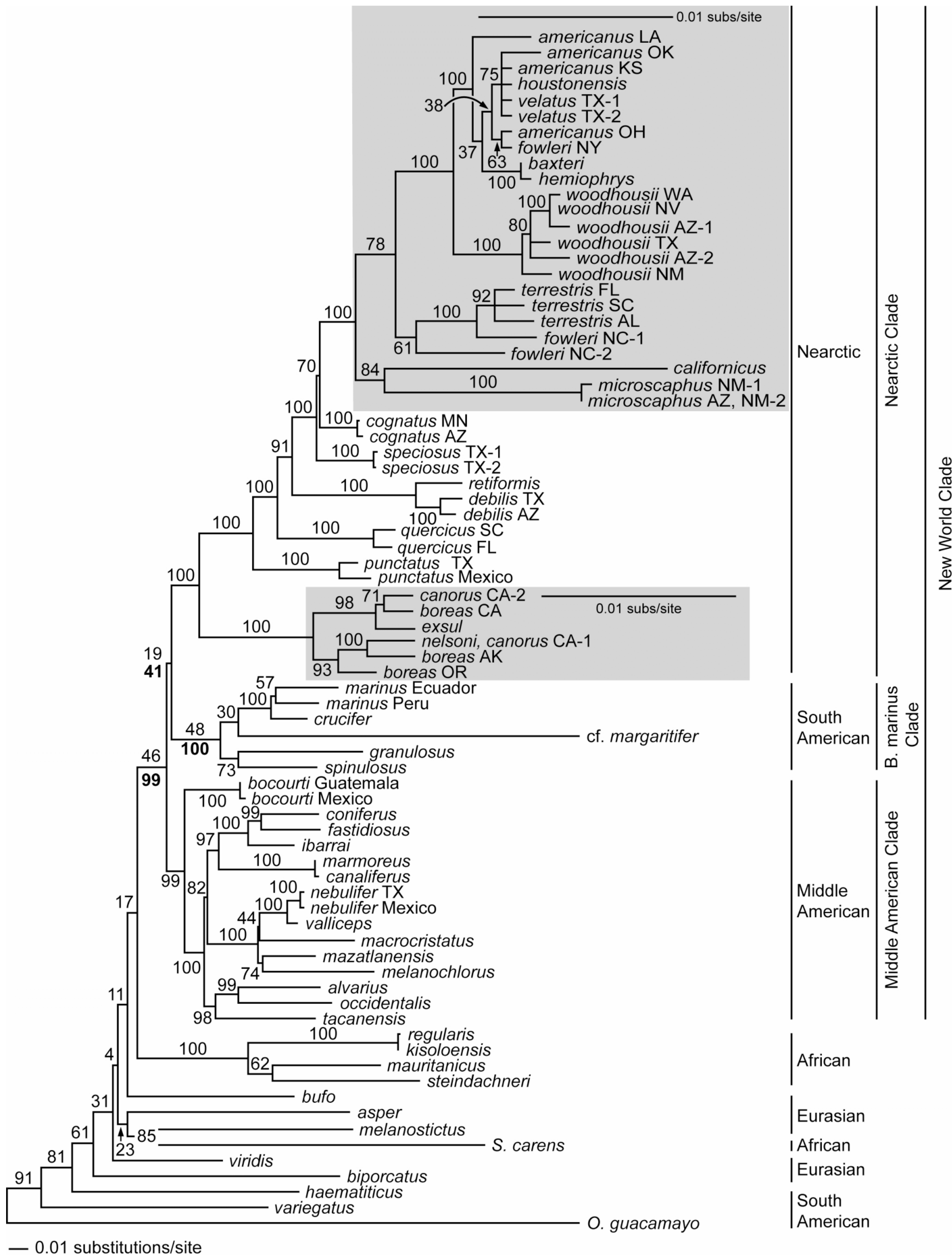


FIGURE 1.2. Maximum likelihood topology. Numbers are the Bayesian posterior probabilities from 450,000 sampled trees. Bold numbers are Bayesian posterior probabilities if *B.* cf. *margaritifer* is excluded (see Results). Outgroup taxa not shown. Branches in shaded boxes are drawn ten times longer than those in the rest of the tree so that the resolution can be seen.

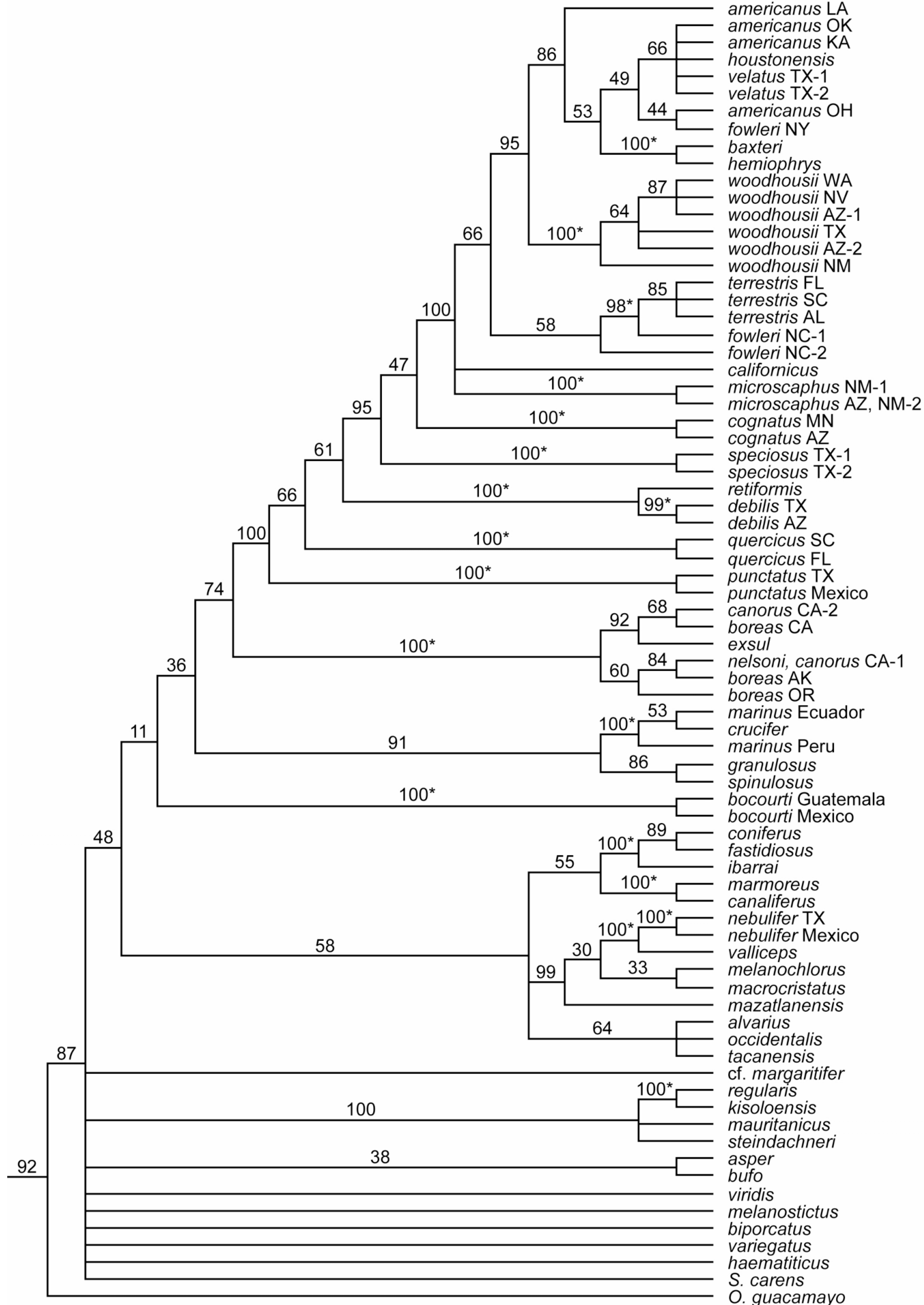


FIGURE 1.3. Strict consensus of the six most-parsimonious trees (CI = 0.336, RI = 0.624). Non-parametric bootstrap support values associated with each node are given as percentages of 1000 pseudoreplicates. An asterisk denotes clades constrained as monophyletic in the likelihood search (see Materials and Methods). Outgroup taxa not shown.

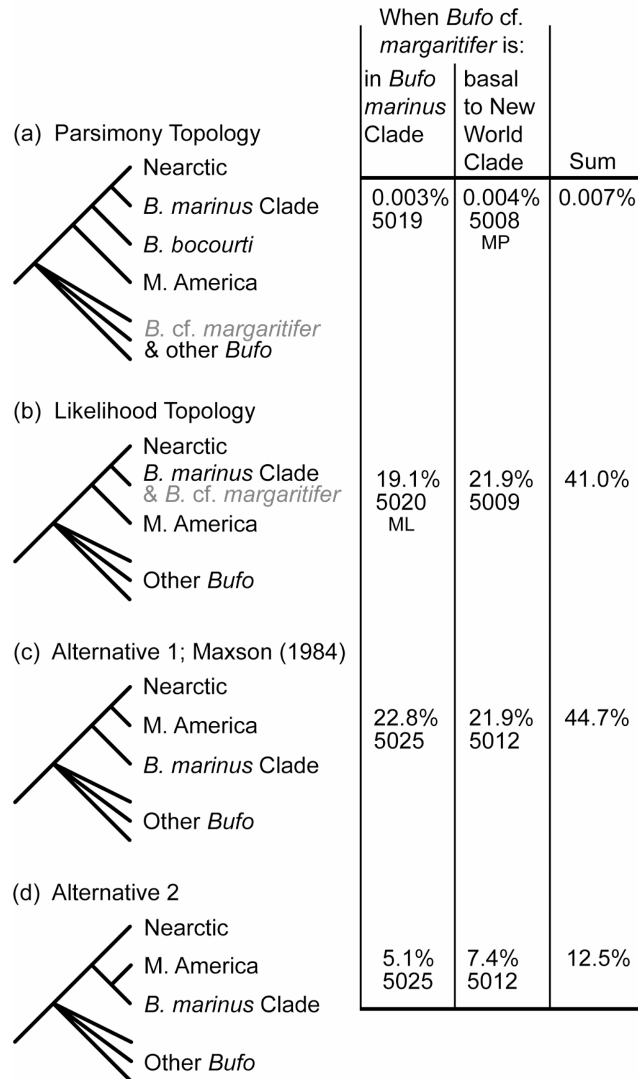


FIGURE 1.4. Bayesian support and parsimony tree length of different topologies within the New World Clade. Relationships among members of each of the three New World clades (the Nearctic, Middle American, and *B. marinus* Clades) were not considered for determining similarity between the Bayesian and 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 *B.* 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.

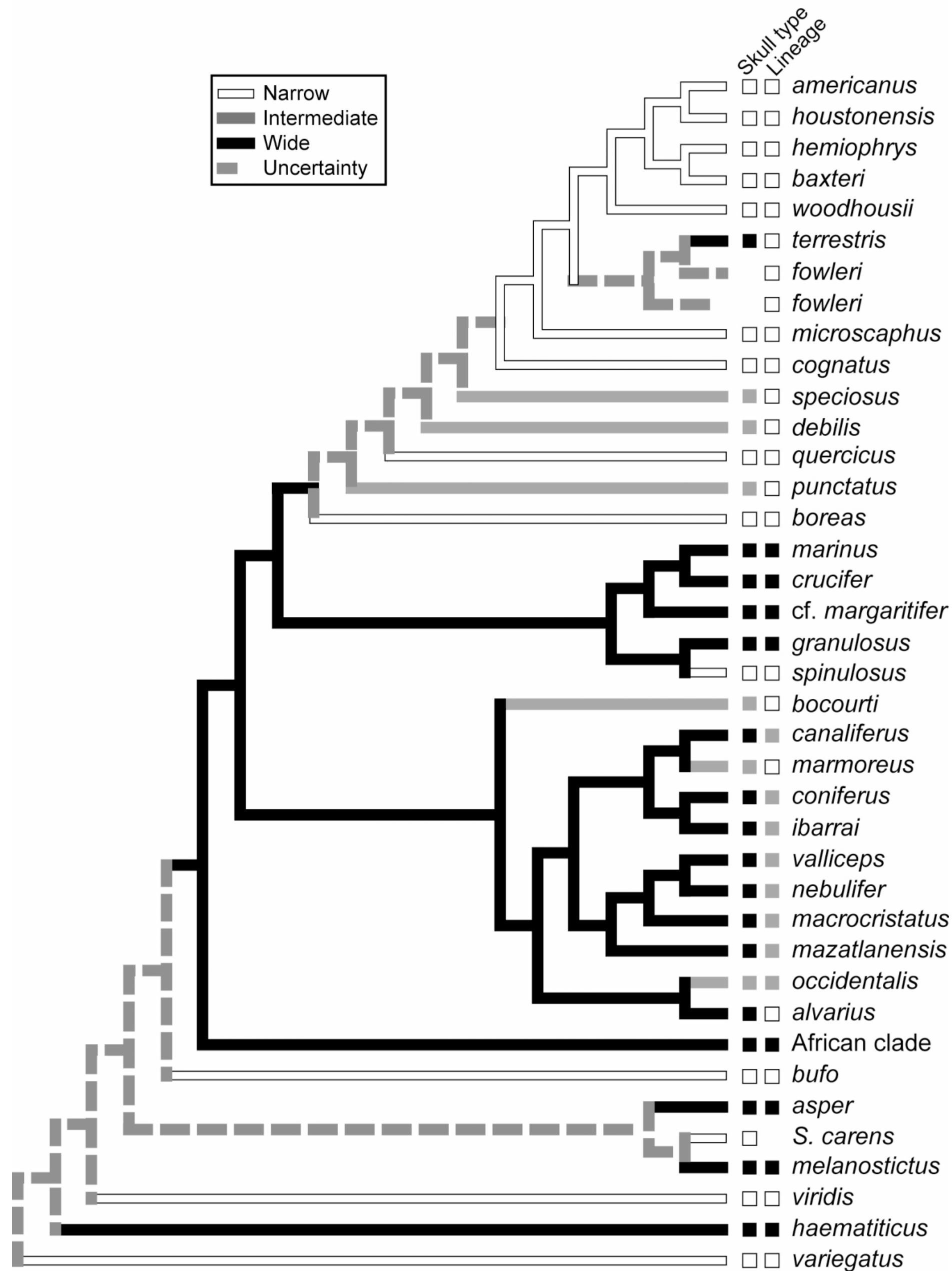


FIGURE 1.5. Reconstruction of the evolution of frontoparietal/skull type on a condensed topology from the maximum likelihood analysis. The first column of squares at the tips of the branches is frontoparietal/skull type (following Martin, 1972); the second column of squares represents the assignment 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.

Chapter 2: Among-population Variation in the Presence of Advertisement Calls in the Western Toad, *Bufo boreas.*

2.1 Introduction

Variation among individuals in their mating signals may impact mating success, and understanding this variation is critical to the study of sexual selection (Darwin, 1871; Kirkpatrick and Ryan, 1991; Andersson, 1994). Some of the most dramatic examples of variation in male mating signals are species exhibiting *within-population* variation in the presence of the primary signal (e.g., Cade, 1981; Zuk et al., 2006). Within a population, some males produce the signal while others do not; these non-signaling males instead rely on alternative mating tactics such as active searching, sneaking copulations, or acting as "satellites" of signaling males (reviewed in Cade, 1980; Gross 1996). One could also imagine a scenario in which variation in the presence of the primary signal exists *among-populations* of one species. Under this scenario, males in some populations produce the mating or advertisement signal while conspecific males in other populations do not produce the signal and rely on alternative strategies to acquire mates. Such a scenario might result from variation in natural selection pressures on signaling males. In populations where large numbers of predators or parasites are also attracted to signaling males, natural selection would favor abandonment of the signal; in populations with

lower predation or parasite risks, sexual selection by female choice would promote continued use of the signal.

To date, there are no documented cases of among-population variation in the presence of the primary mating signal, although there are several species with similar biologies. For example, the primary sexual signal of the Australian and Pacific Islands field cricket, *Teleogryllus oceanicus,* is song, but recent studies have demonstrated the rapid spread of a mutated wing morphology that has resulted in over 90% of males in Kauai populations being unable to produce song (Zuk et al., 2006). These silent males have adopted a satellite strategy in which they congregate near the few remaining calling males in hopes of intercepting phonotaxing females. The spread of this mutated wing morphology appears to be driven by parasite pressure, as the Kauai population has the highest parasitism rates from the acoustically orienting parasitoid fly *Ormia ochracea* of any populations examined (Zuk et al., 1993, 2006). Thus, in *T. oceanicus,* there is among-population variation in the proportion of calling males, but the complete absence of the call has not yet been documented.

Among-population variation in the presence of a cue assessed by reproductive females has also been observed in the Pygmy Swordtail, *Xiphophorus pygmaeus* (Morris and Ryan, 1995; Morris et al., 1996). Females prefer larger males, but large-bodied males only occur in some but not all populations. Although there is among-population variation in the presence of large-bodied males, this trait is a cue and not a true mating signal because it did not evolve to send information. Here, I will examine whether there is

among-population variation in the presence of the major mating signal, the advertisement call, in the Western Toad, *Bufo boreas.*

In most frogs, species and mate recognition is controlled largely by acoustic communication (Blair, 1964a; Straughan, 1973; Wells, 1977; Gerhardt, 1994; Ryan, 2001; Gerhardt and Huber, 2002). The male advertisement call attracts females and announces presence or territory ownership (Wells, 1977; Duellman and Trueb, 1986). Despite conspicuous, species-specific advertisement calls being the rule, there are species that either lack an advertisement call, produce only a feeble one, or have populations consisting of some males that call and others that do not (Martin, 1972; Duellman and Trueb, 1986). There are, however, no known species in which males in some populations call and males in other populations are mute.

Bufo boreas is a likely candidate for among-population variation in the presence of the advertisement call because advertisement calls have rarely been reported in this species, despite the ubiquity of such calls in other species of Nearctic *Bufo.* In general, *B. boreas* is considered to lack a true advertisement call that is comparable to the loud, long, trilled calls of most other *Bufo* including its sister taxon, the Yosemite Toad, *Bufo canorus* (Storer, 1925; Stebbins, 1985; Blair, 1972e; Karlstrom, 1962; Werner et al., 2004). Calls similar to the advertisement calls of many *Bufo* species, however, have been twice reported in *B. boreas.* Cook (1983; p. 63) reported that male *B. boreas* in eastern Alberta have a distinct advertisement call, and Awbrey (1972) noted a single individual (SDNHM 65671) that he identified as *B. boreas* emitting advertisement calls at a site in San Diego County, California. Both authors interpreted these calls to be advertisements

because of their acoustic similarity to typical *Bufo* advertisement calls. No phonotaxis tests, however, were conducted to demonstrate a true advertisement function (i.e., that females can recognize and respond to the call). Throughout this work, "advertisement" calls (in quotes) will be used to indicate hypothesized advertisement calls; these are long, pulsed calls that are acoustically similar to typical *Bufo* advertisement calls but for which the actual function has not been tested through phonotaxis trials.

Nearly all anuran species that produce advertisement calls also have vocal sacs, except some species that call under water (Martin, 1972; Duellman and Trueb, 1986; Dudley and Rand, 1991; Pauly et al., 2006). Vocal sacs are balloon-like pouches that expand with pulmonary air during calling. Inflation of a vocal sac results in an increase in call amplitude (Martin and Gans, 1972), and vocal sacs are considered necessary for producing the loud, long calls of most *Bufo* (Martin, 1971, 1972). Awbrey (1972) noted that SDNHM 65671 possessed a vocal sac, although, as with the advertisement calls, *Bufo boreas* is also generally thought to lack a vocal sac (Storer, 1925; Stebbins, 1985; Russell and Bauer, 1993). Liu (1935), however, reported vocal sacs as absent from his southern sample of *B. boreas* but present from his northern sample, although the voucher numbers, localities, and numbers of individuals examined were not reported.

At present, the available observations and data suggest that some male *B. boreas* may produce calls that are similar to typical *Bufo* advertisement calls. Further, the presence of vocal sacs may also vary across this species' range. However, the pattern of variation both within and among-populations of *B. boreas* has not been documented. To test whether there is among-population variation in the presence/absence of

advertisement calls in *B. boreas,* I conducted call surveys during the breeding season in numerous populations. Release calls and "advertisement" calls were recorded in *B. boreas* and compared to recently recorded advertisement calls of *B. canorus.* In addition to the call surveys and recordings, museum specimens were also examined from throughout the ranges of all species in the *B. boreas* group for the presence of vocal sacs. In examining call production in *Bufo,* Martin (1971, 1972) concluded that vocal sacs are necessary for producing the long, high-amplitude advertisement calls typical of this genus. Thus, absence of vocal sacs can be used to identify populations incapable of producing typical advertisement calls. Finally, phonotaxis tests were conducted at one population where males were found to produce "advertisement" calls to test whether this call serves as an advertisement. Results suggest a complex and previously undocumented history of mating signal evolution.

Study Taxon

The *Bufo boreas* species group is the sister taxon to all other members of the Nearctic clade of *Bufo* (Pauly et al., 2004). This group contains four species: the widespread western toad, *B. boreas*; the Yosemite toad, *B. canorus,* endemic to the central Sierra Nevada of California; and *B. exsul* and *B. nelsoni*, which are each restricted to small valleys east of the Sierra Nevada in eastern California and western Nevada, respectively. *Bufo boreas* occurs from northern Baja California to Alaska and eastward to Alberta and the Rocky Mountains (Fig. 2.1). Phylogenetic relationships within this group reflect apparent conflict between mitochondrial (Graybeal 1993; Goebel 1996; Shaffer et

al. 2000; Pauly et al. 2004) and nuclear DNA (Feder 1977; Pauly, unpublished data). However, the nuclear DNA studies clearly demonstrate that *B. canorus* is the sister taxon to the remaining three species. Further, mtDNA sequence analyses (Goebel 1996; Pauly et al., 2004) and re-analysis of Feder's (1977) allozyme data (Pauly, unpublished data) indicate that *B. exsul* and *B. nelsoni* are nested within *B. boreas,* a result consistent with the long-held interpretation that they are relatively recently isolated populations (Myers, 1942; Schuierer, 1961, 1962; Karlstrom, 1962).

Within the *B. boreas* group, only *B. canorus* is clearly documented to have an advertisement call (the species was even named for its call; "canorus" means "tuneful" in Latin). All 18 Nearctic species outside of the *B. boreas* group produce an advertisement call. All Nearctic species (and most other *Bufo*) that produce advertisement calls have relatively loud calls consisting of numerous pulses, except for the diminutive Oak Toad, *B. quercicus*, which produces loud tonal chirps. All of these species, including *Bufo boreas,* also emit release calls. These are a second common type of vocalization that are generally much quieter than advertisement calls and function to prevent prolonged amplexus (i.e., the anuran mating position) by con- and heterospecific males. Release calls in toads usually consist of a small number of pulses or chirps. Males typically emit these calls after physical contact with others. In *B. boreas,* however, males will also emit calls that sound like release calls but without tactile stimulation (Karlstrom, 1962; Pauly, pers. obs.). Authors who have suggested that *B. boreas* produces a call are referring to this quiet release call-like vocalization that sounds like the repeated peeping of a young chick (Karlstrom, 1962; Martin, 1972; Russell and Bauer, 1993; Storer, 1925; Werner et

al., 2004; Stebbins, 1985). Importantly, such statements should not be interpreted to mean that *B. boreas* produces an advertisement call, as the function of this unique call has never been tested. This call may be a signal to other males as in typical release calls or it may serve some unknown additional purpose. Other authors have recognized the lack of the loud, long, pulsed call typical of other Nearctic *Bufo* and have stated that there is no advertisement call in *B. boreas* (e.g., Blair, 1972e).

2.2 METHODS

Advertisement and release calls

Populations of *B. boreas* and *B. canorus* were visited during the breeding season during spring 2003, 2004, 2005, and/or 2006. Release calls were recorded at all populations, and long, pulsed calls were recorded at populations where these calls were found. For this study, long, pulsed calls were analyzed from five populations of *Bufo boreas* and two populations of *Bufo canorus* (Table 2.1). Release calls were analyzed from three populations of *Bufo boreas* and two populations of *Bufo canorus* (Table 2.1). The release calls are from the same individuals for which advertisement calls were recorded except that the release call recordings for one *B. boreas* had too much background chorus noise for analysis, so release calls of a different individual from the same population were substituted. Calls were recorded using a Sony Walkman (TCD-D8) professional digital audio tapecorder (DAT; at a sampling rate of 48 kHz) and a Sennheiser SE 66 microphone with K3-U power module and a windscreen. At each site, I

attempted to record at least five males and at least five advertisement and release calls per male. Advertisement calls were recorded while holding the microphone between 20 and 80 cm from the calling male. Immediately after calling, males were captured by hand and a body temperature was promptly taken with a Miller and Weber quick-reading cloacal thermometer. Body mass and snout-vent length (SVL) were also measured. Release calls were elicited by simulating male-male amplexus. This was done by gently grasping the male toad in the thoracic region immediately posterior to the forelimbs while holding the microphone within 20 cm of the toad. This is a standard approach for obtaining release calls and yields calls that are not statistically different from conspecific-induced release calls in the congener *Bufo americanus* (Leary, 1999), which produces release calls in the same manner as *B. boreas*. Following recordings of the release calls, body temperature was again taken in case handling impacted toad body temperature. Calls were then digitized using Cool Edit Pro, version 2 (Syntrillium Software) at a sample rate of 44.1 kHz and 16 bits/sample.

Twelve call characters were assessed using the program SIGNAL (Engineering Design). Definitions for the characters and descriptions of how they were measured are provided in Table 2.2 and Figure 2.2. Many of these characters were also examined by Cocroft and Ryan (1995) in their study of call variation in the *B. americanus* and *B. cognatus* species groups. Call rate was also determined for the advertisement calls by measuring the time from the beginning of the first call in a call bout to the beginning of the last call of the same bout in CoolEdit. Because call rate for release calls is presumably a function of tactile stimulation by the amplexing males, release call rate was not

assessed. Pulse type, a character used intensively by Martin (1972) in his analysis of bufonid advertisement calls, was also determined by examination of waveforms directly in CoolEdit for the presence of interior amplitude modulation of pulses.

In the typical *Bufo* pulsed advertisement calls, the frequency and amplitude of pulses increase during the early part of the call and then stabilize. This early portion of the call is termed the initial transient. The duration of this transient was termed the call rise time by Cocroft and Ryan (1995), and the difference in dominant frequency of pulses at the start and end of the transient was used as the frequency modulation within the call. For *B. canorus* and *B. boreas,* it was difficult to identify a repeatable definition for detecting the end of the initial transient. Therefore, call rise time was measured as the duration from call onset to the first pulse having ≥75% of the maximum amplitude of the call (Fig. 2.2). Frequency modulation of the call was then measured as the lowest dominant frequency of any pulse from a series of 10 pulses from the middle of the call minus the dominant frequency of the first pulse. These 10 pulses make up the mid-call transect, which generally occurs well after frequency and amplitude have stabilized among pulses (Fig. 2.2). In rare instances, the first pulse had a frequency greater than any other pulses in the rest of the call (i.e., a high frequency initial pulse) and such pulses were excluded from this measurement. Additionally, in a small number of cases (seven *canorus* calls and four *boreas* calls) the dominant frequency of the first pulse was identical to the frequency of some mid-call pulses and greater than the minimum dominant frequency of one of the mid-call pulses. Such differences were reported as 0 instead of as small negative values to indicate that the frequency of the initial pulse was

equivalent to the frequencies of pulses from mid-call (i.e., there was no initial transient for frequency). Pulse rate, pulse duration, pulse rise time, pulse shape, pulse duty cycle, and dominant frequency were all measured as the average of these values from the 10 pulses of the mid-call transect (Table 2.2). Call rise time and frequency modulation could not be measured in the Montana population because males had a relatively low amplitude call, and the initial pulses of the call were indistinguishable from the background.

As with most other Nearctic *Bufo,* release calls of *B. canorus* and *B. boreas* have far fewer pulses than advertisement calls and also lack an initial transient that includes numerous pulses. Therefore, no features of the initial transient (i.e., call rise time and frequency modulation) were measured. However, the first pulse of the release call is often shorter and of lower amplitude than subsequent pulses and has a frequency occasionally outside of the range of later pulses in the call. Therefore, pulse rate, pulse duration, pulse rise time, pulse duty cycle, and dominant frequency were measured as the average from all pulses in the release call except the first pulse. Because there is no interpulse interval associated with the final pulse, interpulse interval and pulse shape were measured for all but the first and last pulses in the release calls. So that the reported values are averages from at least two pulses per release call (except for interpulse interval and pulse shape), all release calls analyzed had at least three pulses. These toads do produce release calls with only one or two pulses, so this selection criteria upwardly biases the measurement of pulse number.

For each call type, call characters were assessed for five calls per individual, except in a small number of individuals for which fewer calls were available. These

values were averaged across the advertisement or release calls of each individual and the averages used to assess correlations with temperature and body size. Separate regressions were conducted for each call type and each taxon in SYSTAT (SYSTAT Software Inc.). Characters found to be significantly associated with temperature ($P < 0.05$) were standardized to 18°C to facilitate comparisons among *boreas* and *canorus*. This temperature was chosen because it is within the relatively narrow range of overlap between the recordings of the two species (*B. boreas* populations 1–4: mean 14.8°C, range 9–19.4°C; *B. canorus* mean = 22.1°C, range 17–24.6°C). The "advertisement" calls produced by individuals in population 5 are quite different from the calls produced by the Alberta populations (pops. 1–4). Unfortunately, calls of only three individuals were recorded, which is an insufficient number to allow for separate regression analyses for this population. However, the three recorded individuals had body temperatures of 16.4, 18.8 and 20.2°C (mean = 18.4°C). Because the mean value is so close to 18°C, values reported for mean call characteristics are unlikely to be significantly different than values that would be recovered at 18°C for any of the traits that would vary with temperature based on a larger sample. Therefore, these values are reported without any temperature corrections.

The amplitude (sound pressure level; SPL) of advertisement calls was also determined by using a digital sound level meter (RadioShack Model No. 33-2055) set to C weighting and fast response. The measured decibel (dB) level and the distance to the calling male were documented and values were subsequently standardized to 0.5 m.

Vocal sacs

In nearly all species of anurans, production of advertisement calls is accompanied by the inflation of a vocal sac (Duellman and Trueb, 1986). These secondary sexual traits have not been documented in females (even in species where females produce calls; Schlaepfer and Figeroa-Sandí, 1998) or in juvenile males. Anuran vocal sacs are of three types: single median subgular sacs, paired median subgular sacs, or paired lateral sacs (Liu, 1935). In bufonids, the vocal sacs are of the single, median subgular type, although during development two separate sacs may be present, one extending from each vocal slit, that eventually fuse medially to form a single, contiguous sac. In bufonids and most other anurans, vocal sacs result from a diverticulum of the lining of the buccal cavity that extends ventrally through one or two small apertures in the floor of the buccal cavity (i.e., vocal slits). This evagination forms a pouch or sac within the underlying submandibular musculature (principally the interhyoideus muscle [Fig. 2.3]; muscle terminology following Tyler 1971, 1974; see also Pauly et al., 2006). Inflation of the vocal sac stretches these submandibular muscle(s) and surrounding gular skin, which may or may not be strongly modified, resulting in the distensible, balloon-like structures that are often apparent to observers of calling anurans.

To assess the presence of vocal sacs in the *Bufo boreas* group, I examined preserved specimens of the four recognized species of this group. Efforts were focused on *B. boreas* from Alberta, eastern British Columbia, Montana, and southern California because specimens from Alberta and southern California had been reported to have "advertisement" calls (Cook, 1983; Awbrey, 1972). Further, because Awbrey's (1972)

observation was from a site where *B. boreas* and the Arroyo Toad, *B. californicus,* are sympatric, I especially concentrated on regions where these two species co-occur. Finally, a small number of previously identified *B. boreas* x *B. punctatus* and *B. boreas* x *B. canorus* hybrids were also examined (Feder, 1979; Morton and Sokolski, 1978). Only adult males were examined for vocal sac presence; sexual maturity was determined by the presence of nuptial pads on the fingers (Inger and Greenberg, 1956; Duellman and Trueb, 1986).

Specimens included individuals collected as part of this research (and now in the Texas Natural History Collection) as well as specimens made available through the following institutions: California Academy of Sciences (CAS); Carnegie Museum of Natural History (CM); Cornell University Museum of Vertebrates (CU); Royal British Columbia Museum (RBCM); the National Museum of Natural History (USNM); San Diego Natural History Museum (SDSNH); United States Geological Survey, National Wildlife Health Center (USGS-NWHC); University of Alberta, Museum of Zoology (UAMZ); Museum of Vertebrate Zoology, University of California, Berkeley (MVZ); University of California, Davis, Museum of Zoology (UCDMZ); University of Kansas Natural History Museum and Biodiversity Research Center (KU); and the Texas Natural History Collection, University of Texas, Austin (TNHC).

Examination of specimens typically involved opening the mouth and using a blunt dissecting probe to examine the buccal floor for the presence of vocal slits. The number of vocal slits (0, 1, or 2) was then recorded as was the side if only one was present. The vocal slits are immediately lateral to the ceratohyal (i.e., the anterior cornu of the hyoid,

sensu Tyler 1971, 1974). In some individuals lacking vocal slits, the buccal floor in the vicinity of the ceratohyal is very thin and even gentle examination with a dissecting probe can infrequently result in small tears that could be misidentified as vocal slits. Similarly, in some specimens, degradation of the buccal cavity can make positive identifications of vocal slits challenging. For specimens in which the presence of vocal slits was not obvious, presence/absence of the vocal sac was determined by making a medial incision in the gular skin allowing direct examination of the submandibular musculature. Vocal sac occurrence was then determined by the following suite of characters (Fig. 2.3): 1) posterior development of the interhyoideus including a pouch-like appearance rather than being a single transverse sheet; 2) retraction of the median raphe anteriorly and the associated elongation of the muscle fibers of the interhyoideus to form (nearly) contiguous transverse fibers largely spanning between mandibles; and 3) the appearance of elastic connective tissues between the elongated muscle fibers of the interhyoideus. These structures are all associated with vocal sac development and are not found in juvenile males or females (Fig. 2.3; Jaramillo et al., 1997; Tyler, 1971, 1974; Pauly, unpublished data). Tests for vocal slit side bias and for differences in body size between individuals with one or two slits were conducted in SYSTAT.

Phonotaxis tests

Phonotaxis testing was conducted at Meanook Biological Research Station (Population 2) to test whether the long, pulsed call emitted by males in the Alberta populations functions as an advertisement call. Females must be able to recognize and

respond to the call for it to be an advertisement call. Testing was conducted in a chamber made with a PVC pipe frame and walls of acoustic absorbance foam. Internal dimensions of the chamber were 180 cm x 140 cm and approximately 100 cm tall. Speakers were placed in the two corners of a long wall opposite of each other and at least 10 cm out from the wall allowing toads to move around the back of the speakers. A cone was placed along the center of the other long wall. The distance from the cone to each speaker was 125 cm and the two speakers were 145 cm apart. All testing was conducted indoors in a completely darkened room to avoid temperature fluctuations and sound and light nose, such as moonlight, background chorusing, and wind noise (reviewed in Gerhardt, 1992). Trials were filmed under infrared light and viewed on a video screen in an adjacent room. Speaker choice and time to choice or experiment end were recorded for all trials.

Test stimuli were natural calls recorded from Population 2 males. Calls were recorded in June 2004 and May and June 2005. A representative call from each male was used to make a test stimulus that was broadcast at call rate of 2.57 calls per minute or 1 call within each block of 23.3 seconds. This rate is the average call rate of 10 individuals recorded in 2004. Several temporal characters of *B. boreas* calls are correlated with temperature. Therefore, in most cases the test call used was selected so that the temperature of the recorded male and the chamber temperature were within 1°C. For some chamber temperatures, appropriate calls were not available so calls were modified to have temporal characteristics appropriate for the testing chamber. This was done by modifying the tempo of the call in CoolEdit (using the time stretch command) according to the regression equation for pulse rate and temperature. This correlation was determined

by measuring pulse rate from five calls from 16 individuals of *B. boreas* from the Meanook population. An average pulse rate for each individual was calculated and plotted against temperature. The resulting regression line was $y = 0.5335X - 1.1619$ ($r = 0.9672$, $P < 0.001$).

The phonotaxis trials were recognition tests in which one speaker broadcast white noise (0–5000 Hz) while the other broadcast the hypothesized advertisement call from a local male. The duration and broadcast rate of the noise was identical to that of the call being used for that trial. The noise and test call were broadcast antiphonally and at the same sound pressure level. If females are able to recognize and respond to the call, females motivated to breed should go to the call speaker. However, if the call is not an advertisement, then approaches to the speakers should be incidental and in approximately equal frequency to both speakers. To prevent side or direction biases, the speaker broadcasting the test signal was selected at random. Once a call elicited a response, it was not used in subsequent tests.

For each trial, a female captured at a breeding pond was placed under the cone in the phonotaxis chamber. Test stimuli were then broadcast at 72 db SPL (re 20 micro Pascals) at 50 cm. This value represents a common amplitude for *B. boreas* calls (see Results). The decibel level was checked prior to every test. After 3 minutes, the cone was raised allowing the female to move about the chamber. Speaker choice was determined by a female moving towards a speaker and approaching within 10 cm of the front of the speaker. Approaches to the side or back of the speaker were not counted until the female moved to the front of the speaker. As a result, a choice was not counted if a female

approached to within a few centimeters of the speaker simply by walking along the chamber wall. The trial would end, and therefore no choice was counted, if the female did not move in the initial 5 minutes, did not move for 2 minutes after any initial movement, if no choice was made within 15 minutes, or if she attempted to climb the walls of the chamber two times.

2.3 RESULTS

Call surveys and analyses

Eighteen *B. boreas* sites were visited during the breeding period. Sites were in California (7), Washington (2), Montana (3), Alberta (5), and Alaska (1). The production of long, pulsed calls similar to typical *Bufo* advertisement calls was documented at the five Alberta localities and one Montana locality (Figs. 1, 4). No long pulsed calls were heard at any other sites, including the site where Awbrey (1972) reported a calling *B. boreas* (Pine Valley) and a neighboring valley with an even larger *B. boreas* population (Sweetwater River, Descanso, San Diego County, California). At the six sites, "advertisement" calling was unmistakable and multiple individuals contributed to prolonged chorusing activity. Calling was most pronounced at night but also occurred during the day during the peak of the short breeding period (often 2–5 days). At the five Alberta localities (Table 2.1, populations 1–4 and an additional site on Hwy 88, near the northeast corner of Lake Utikuma; N 55.93360°N, 115.16907°W; Fig. 2.1), numerous

males emitted loud, long calls, and "advertisement" Calling was accompanied by the inflation of an obvious vocal sac.

At the single population in Montana where calling was observed, males routinely emitted long, pulsed calls (Fig. 2.4), but these calls were much quieter than observed at the Alberta locality. There was no externally apparent vocal sac (nor were vocal sacs found upon inspection of preserved specimens; see below). Males producing these unique calls behaved similarly to males producing the louder calls in the Alberta localities and to *B. canorus*. Males called from the shore or by climbing on top of or holding onto vegetation mats and logs. Calling males assumed the typical calling posture of toads with forearms mostly extended so that the anterior portion of the body is well off the substrate.

Call amplitude (dB SPL) was measured for 12 calling males in Alberta (*B. boreas* populations 1, 2, and 4), 1 male from Montana (population 5), and 1 male *B. canorus* (population 1). Measurements were taken 20 to 50 cm from the calling male and were subsequently standardized to 50 cm. In the calling *B. boreas* with vocal sacs, call amplitude at 50 cm averaged 70.7 dB (range 66–76 dB SPL). In contrast, the single male measured from the Montana population was much quieter at 50 dB SPL at 10 cm, which is equivalent to 36 dB SPL at 50 cm. This low value is not a result of this one male being an outlier because numerous other males in this population were observed to have similar amplitude calls and no males in the Montana population were ever observed to produce calls as loud as those heard at the Alberta sites. Differences in amplitude of the calls were also easily observable as differences in their transmission distance. In the field, I could detect calls from active choruses at distances greater than 650 m at Alberta localities, but

only up to approximately 50 m from choruses at *B. boreas* population 5 in Montana. The calls of *B. canorus* were of similar amplitude as to the Alberta *B. boreas;* the single individual measured was calling at 72 dB SPL at 50 cm.

From each population, recordings of five calling males were used for analyses with five calls analyzed per individual when possible (Table 2.1). In the Montana *B. boreas* population, recordings could only be obtained from three individuals. Three additional individuals were also analyzed from *canorus* population 1 to increase the temperature range of the *canorus* sample for use in regression analyses. In total, 97 Alberta and 15 Montana *boreas* "advertisement" calls and 67 *B. canorus* advertisement calls were analyzed. A similar sample was analyzed for the release calls, except that release calls from *boreas* populations 3 and 4 were not used.

In both *B. boreas* and *B. canorus*, each pulse results from a single contraction of the thoracic musculature pushing a pulse of air through the larynx and vibrating the vocal cords. Therefore, a number of temporal call and pulse characters are expected to be correlated with temperature. At higher temperatures, muscles may be contracted more quickly resulting in a faster pulse rate, faster pulse rise time, shorter pulse duration and interpulse interval, and, consequently a shorter call duration than calls of a similar number of pulses at lower temperatures. As expected, pulse rate was positively correlated with temperature for the *B. boreas* "advertisement" calls (populations 1–4). Call duration, pulse duration, interpulse interval, pulse rise time, and call rise time were negatively correlated with temperature (Table 2.3). For the *B. canorus* advertisement calls, pulse rate was again positively associated with temperature, and pulse duration and interpulse

interval had a negative association (Table 2.3). For the release calls, *B. boreas* again had a larger number of traits with significant correlations with temperature (Table 2.3). These correlations were used to standardize individual averages to 18°C.

Comparisons of calls of Alberta *B. boreas* with *B. canorus*

Call duration for the high-amplitude, long, pulsed calls averaged twice as long in *boreas*, which also emits more pulses per call (Table 2.4). Further, the pulse rate is slower in *boreas*, and *boreas* has slightly longer pulses and a much longer interval between pulses. As a result, *boreas* has a lower duty cycle.

Martin (1972) classified the pulses of *Bufo* calls into three types based on the pattern of amplitude modulation within pulses. The waveforms of Type III pulses are somewhat symmetrical with a relatively long rise time. Type II pulses have a repeated pattern of interior amplitude modulation in which each pulse consists of several sub-pulses. Type II pulses typically have a faster rise time because an early sub-pulse usually has the greatest amplitude. The Alberta *B. boreas* usually have Type III pulses in their "advertisement" calls (Table 2.4, Fig. 2.4), although 3 of the 20 males recorded had pulses more consistent with Type II modulation. The pulses of Alberta *B. boreas* release calls, *B. canorus* release calls, and *B. canorus* advertisement calls show Type II modulation (Table 2.4, Fig. 2.4). However, 4 of the 13 *B. canorus* had pulses with minimal interior amplitude modulation that were more similar to the Type III pattern. Because Type III pulses have a slower rise time, pulses of the *B. boreas* "advertisement"

call had a slower rise time and a slightly higher value for pulse shape than pulses of the *B. canorus* advertisement calls.

The dominant frequencies of the two species' calls are similar but are more divergent when taking into account body size. Both species show significant correlations with body size (*boreas:* y = -9.95X + 2046.89, r = 0.472, P = 0.036; *canorus:* y = -25.87X + 2927.38, r = 0.680, P = 0.011). When standardized to the same body size, the DF of *boreas* is approximately 100 Hz greater than that of *canorus* (e.g., at 62mm SVL, *boreas* calls at 1430.3 Hz and *canorus* at 1323.4 Hz). Dominant frequency was also found to be correlated with temperature in *boreas* but not *canorus*. Correlations between dominant frequency of calls and temperature are rare for anuran advertisement calls but were also found in *B. americanus* (Zweifel, 1968). However, regardless of whether the regression equation is used to standardize the *boreas* calls to 18°C, the dominant frequencies of *boreas* and *canorus* calls are similar (mean DF for *boreas* = 1370.6 Hz; mean DF for *boreas* standardized to 18°C = 1418.4 Hz; mean DF for *canorus* = 1393.1 Hz) except when body size is incorporated as above.

The release calls are very different from the longer pulsed calls in both species (Table 2.4, Fig. 2.4). Release calls have far fewer pulses (≤11) and are much shorter than the long pulsed calls, almost always lasting no more than two seconds in *B. boreas* and even less in *B. canorus* (Table 2.4). The pulse rates are lower for the release calls, which is entirely due to their longer interpulse intervals. Pulse shapes in the release calls are also much smaller because the highest amplitude of each pulse is reached very quickly (i.e.,

they have a short pulse rise times; Fig. 2.4). The variation in the peak amplitude of subsequent pulses is much greater in the release calls than in the advertisement calls.

The release calls of the Alberta *B. boreas* and *B. canorus* are more similar than their advertisement calls. Pulse shape, pulse duty cycle, and the number of pulses/call are very similar (Table 2.4). However, the pulse rate of the release calls is lower in the Alberta *B. boreas.*

Comparisons of calls of Alberta *B. boreas* with Montana *B. boreas*

The "advertisement" calls from the Montana population were slightly shorter and with fewer pulses than those recorded in the Alberta populations (Table 2.4). However, this difference is overestimated because these calls are so quiet relative to the Alberta calls that the first few pulses often could not be detected on the recordings. Call rates were also similar in the Montana and Alberta populations. Dominant frequency of the Montana "advertisement" calls was much lower, which is expected given the much larger body size of the Montana males. The regression equation for dominant frequency and body size in the Alberta populations could be used to estimate the dominant frequency of Alberta males at the size of Montana males (mean SVL = 91 mm), although this size is well outside of the range observed in Alberta. Nevertheless, a 91 mm Alberta male should call at 1141.9 Hz, which is more than 200 Hz greater than the calls of the Montana males. The same method can be used to assess the release call dominant frequency of a 91 mm Alberta male, which would be 880.6 Hz (DF of the Alberta release call is also correlated with SVL: y = -12.37X + 2006.06, r = 0.747, P = 0.013). This value is

remarkably similar to the observed dominant frequency in the "advertisement" calls of the Montana males. Interestingly, the variation in the peak amplitude of subsequent pulses is also similar between the long "advertisement" calls of Montana males and the release calls of the Alberta toads.

Vocal sacs

Vocal sacs were found in all male *B. canorus* and in some, but not all *B. boreas*. Vocal sacs and slits of both species were of similar size and structure, except for two *B. boreas* from San Diego County, California that had abnormal and incompletely developed vocal sacs (described below).

A total of 1279 adult male toads of the *Bufo boreas* group and 15 adult hybrids were examined for the occurrence of vocal sacs. Because *B. exsul* and *B. nelsoni* have extremely small ranges, only 15 individuals were examined for each species; all *B. exsul* and *B. nelsoni* lacked vocal sacs, as did other *B. boreas* from nearby regions of eastern California and western Nevada. In contrast, all 172 *B. canorus,* which are from at least 24 localities spanning the species' range, had vocal sacs; forty-four individuals had two vocal slits and the remaining had only one vocal slit on either side; there were no obvious patterns of side bias for individuals with only one slit. The two *B. punctatus* x *B. boreas* hybrids and 10 of the 13 *B. boreas* x *B. canorus* hybrids also had vocal sacs.

Vocal sac occurrence in *Bufo boreas* presents a much more diverse pattern (Fig. 2.1). The *B. boreas* sample included 1077 individuals from at least 419 distinct localities from throughout the species' range. All of the *B. boreas* with vocal sacs were restricted to

the northeastern corner of the species range in Alberta and a single population in Montana, except for two individuals from southern California. Of the 124 *B. boreas* found to have vocal sacs, 35 had two vocal slits and the remaining males had only one vocal slit with slits on either side occurring at equal frequencies in the two populations with adequate sample sizes for statistical analysis (Meanook Biological Research Station: $n = 17$; $X^2 = 0.529$, df = 1, $P = 0.467$; Lily Creek at Lesser Slave Lake: $n = 31$; $X^2 = 1.636$, df = 1, $P = 0.201$). A one-tailed t-test was also used to test whether males with two vocal slits are larger than males with one slit in the Lesser Slave Lake population as this was the only population with an adequate sample of individuals with both conditions. This hypothesis could not be rejected ($t = 1.789$, df = 29, $P = 0.958$) suggesting that sexually mature males can have either one or two vocal slits and that possessing one slit is not always an intermediate stage in the development of a vocal sac with two vocal slits.

The Montana population as well as two western Alberta populations are the only *B. boreas* populations in the northeastern section of the range in which adult males with and without vocal sacs occur at the same site. The Montana population is from Hanging Gardens, approximately 1.7 km east of the Continental Divide in Glacier National Park (MVZ 187418, 187423–426, 187430, 187431, and USGS-NWHC 4663-001–003). Only one of ten individuals in this sample lacked a vocal sac. Specimens from four nearby localities (14.2–36.0 km from Hanging Gardens) on both sides of the Continental Divide lacked vocal sacs.

The two Alberta localities that have males with and without vocal sacs appear to be transitional populations between areas where males have vocal sacs to the east and

where they lack them to the west. Of the five specimens from Maskuta Creek in central Alberta (USNM 48620, -24, -26, -28, -30; original collection locality is reported as "Prairie Creek," which is now known as Maskuta Creek, just south of Hinton, Alberta), only one has a vocal sac. The three specimens examined from Brule Lake (UAMZ 2222, 2223, and 2225), 13 km west of Maskuta Creek, lack vocal sacs, but the two specimens that are only 33 km northeast of Maskuta Creek (MVZ 69041, 69042) have vocal sacs. At the second mixed locality, only one of three specimens collected from the town of Demmitt in northwestern Alberta has a vocal sac (UAMZ 102, 687, 688). The four males from Tupper Creek, British Columbia (RBCM 192–195), 9.5 km northwest of Demmitt, and the single male from Moonshine Lake, Alberta (UAMZ 2443), 64 km northeast of Demmitt lack vocal sacs. At sites east and south of Demmitt, males have vocal sacs, although these sites are 270 km away.

In addition to Awbrey's (1972) toad, two *B. boreas* from the vicinity of Descanso, San Diego County, California were also found to have vocal sacs (SDSNH 55333 and 55499). However, the vocal sacs of these individuals were atypical and poorly developed. The vocal slits of the two Descanso individuals were more anterior than in any other *B. boreas* or *B. canorus* examined, and in much the same position as observed in *B. californicus.* There was no or minimal posterior development of the interhyoideus, and the vocal sac was extremely narrow and confined to only one side by the presence of a complete or nearly complete median raphe bisecting the interhyoideus. Admittedly, both specimens were small (63 and 71 mm SVL) relative to *B. boreas* examined from this area (mean = 75.6; range 63–86 mm SVL,) suggesting that the minimal development could be

due to a young age, but they did have nuptial pads. One possibility is that these toads are *B. boreas* x *B. californicus* hybrids. The vocal slit location is more similar to that seen in *B. californicus,* although there were no other obvious morphological traits to suggest that they are hybrids. Specimens from portions of southern California where the ranges of *B. californicus* and *B. boreas* overlap were sampled intensively to determine if vocal sac presence was more widespread. Despite examining 172 specimens from counties where both species are known (including 25 males from Descanso and immediate vicinity), no other males with vocal sacs were found. Further, field surveys were conducted both in the Sweetwater River in Descanso and in Pine Valley, which is the next drainage east of the Sweetwater and is the collection locality of Awbrey's anomalous toad (see below). Both localities are breeding sites for *B. californicus* and *B. boreas.* Twenty *B. b*oreas were also examined along the Sweetwater River in Descanso for vocal slits and then released, but none had them. Additionally, despite nightly surveys during the entire 2005 and 2006 *B. boreas* breeding seasons at these sites, no *B. boreas* were observed producing long, trilled calls (at least 200 individuals were observed) even though surveys continued past the peak of the *B. boreas* breeding and into the later breeding period of *B. californicus*.

The toad from Pine Valley that was identified as *B. boreas* by Awbrey (1972) was also examined by dissecting the gular skin to observe the condition of the submandibular musculature. The interhyoideus and the vocal sac are approximately twice as large as typical *B. boreas* group toads with vocal sacs. The vocal slits are also much larger than those of typical *B. boreas* and *B. canorus,* as is the tympanum of this male. This specimen is clearly not a typical *B. boreas.* Some possibilities are that this animal is a

developmentally abnormal *B. boreas*, a hybrid with *B. californicus,* or an undescribed species. Examination of 12 morphometric traits from 40 male *B. boreas,* 40 male *B. californicus,* and this specimen, followed by multivariate analyses were conducted in hopes of identifying the appropriate classification of this specimen. This male was found to be extremely different from all other males examined (results not shown). Although the appropriate classification remains unknown, this specimen is certainly not a typical *B. boreas* and is not treated as a *B. boreas* with a vocal sac in this study. The two other males with vocal sacs from this region had external morphometric characteristics that were within the range of variation of both *B. californicus* and *B. boreas.*

Phonotaxis tests

In twenty trials, females approached the front of a speaker and were classified as having made a choice. In all of these trials, females approached the speaker broadcasting the call and always made physical contact with the front of the speaker. In most successful responses, females quickly approached the speaker once they were released following the three-minute acclimation period. The average time from release to contact was 176.4 s (range 61–551 s). The majority of movements towards the speaker were made while the call was being broadcast, and the average number of calls from release to speaker contact was 8 (range 3–23).

2.4 DISCUSSION

Bufo boreas has long been considered to lack the distinctive, long, pulsed advertisement call that is common to all other species of Nearctic *Bufo*. However, the work presented here demonstrates that there are two distinct calls in *B. boreas* that are acoustically similar to advertisement calls of other *Bufo* (Table 2.1, Fig. 2.4). Both calls are long and pulsed as is typical of bufonid advertisement calls, but one is of a similar amplitude to other advertisement calls, while the second is of much lower amplitude. This higher-amplitude call is emitted by males with vocal sacs and is unquestionably an advertisement call because females recognize and respond to the call in phonotaxis tests. Further, at population 2, a female was observed to approach the most actively calling male in a chorus of at least 12 males. The female approached the male and contacted his forearm while he called. The male abruptly ended his call and grasped the female in amplexus.

The geographic boundary of populations that produce this advertisement call was inferred by examining specimens for the presence of vocal sacs. The only populations fixed for the presence of vocal sacs and therefore presumably capable of producing this long high-amplitude call are in the northeastern corner of the species range (Fig. 2.1). Populations to the south and west of this region do not have vocal sacs, and no high-amplitude, pulsed calls similar to those heard at the Alberta localities were observed outside of Alberta. The presence of a high-amplitude, long, pulsed advertisement call in a geographically restricted set of populations demonstrates that among-population variation exists in the production of the major mating signal in this species.

The second type of long, pulsed call observed in *B. boreas* is quieter than the Alberta advertisement call and was only observed in a single, large population in northwestern Montana. The function of this call is not currently known. As with the high-amplitude call farther north, it may also function as an advertisement and research is currently underway to examine this. At present, there are no known morphological correlates of call production that would allow the use of museum specimens to determine in which populations males produce this call.

Long pulsed calls, regardless of amplitude, were never heard outside of the Montana and Alberta populations. Although calls acoustically similar to typical toad advertisement calls do not occur in the majority of *B. boreas* populations, the possibility that some calls serve an advertisement function in these populations cannot be ruled out. Calls that are acoustically similar to release calls but emitted without tactile stimulation were observed, although infrequently, at most populations during the breeding season. It is possible that females recognize and respond to these calls and are therefore using these weaker calls for mate identification in the absence of the more typical long, pulsed call.

Bufo boreas is thus the only species known to have among-population variation in the presence/absence of the major mating signal. Because the vocal sac is so critical to advertisement call production in anurans, studies of vocal sac occurrence may identify additional species with among-population variation in the presence of advertisement calls. Previous studies have reported among-population variation in the presence of vocal sacs in several species, but later research found these differences to be between different species. For example, Inger (1954) reported geographic variation in the occurrence of

vocal sacs in southeast Asian *Polypedates leucomystax,* with vocal sacs present in *P. l. quadrilineatus* but absent in *P. l. linki*. However, Inger (1954) treated *P. l. linki* as a junior synonym of *P. macrotis,* which is now regarded as a separate species that is likely not the sister taxon to *P. leucomystax* (R. M. Brown, pers. comm.). Thus, it appears that *P. leucomystax* has vocal sacs while *P. macrotis* lacks them, although further study of species boundaries and vocal sac occurrence is needed for these species. Geographic variation in vocal sac occurrence was also reported in *Rana aurora* (Hayes and Krempels, 1986), a species from western North America, but this variation is now recognized as occurring between distinct species (Shaffer et al., 2004). Interestingly, where these two species come into contact along the northern California coast, there is a long zone (approximately 480 km) in which males often have an “intermediate” condition of asymmetric or rudimentary vocal sacs (Hayes and Krempels, 1986), even though molecular markers suggest a much narrower contact zone (Shaffer et al., 2004). There is also extensive variation in the occurrence of vocal sacs in the *Rana palmipes* species group. This group includes eight species of which some are fixed for the presence of vocal sacs, others lack them, and in the sister species *R. vaillanti* and *R. palmipes* there are males with and without vocal sacs (Hillis & de Sá 1988; Hillis, personal communication). Males with and without vocal sacs, however, occur within (and not among) populations. Thus, at present there are no other species with among-population variation in the presence of vocal sacs but few studies have specifically looked for such patterns.

Why is there geographic variation in the presence/absence of the major mating signal in *Bufo boreas*?

Wells (1977) summarized patterns of anuran mating systems and suggested that for species with high density breeding aggregations and/or short breeding seasons, males typically engage in active searching for females instead of calling from a stationary position. In low-density choruses and/or in prolonged breeders where competition among males for access to females is less, males typically vocalize from a single area and wait for a female to approach. This paradigm predicts that in the Alberta populations the breeding season should be longer and/or densities of males at the breeding sites less than occurs in other parts of the range. However, in all populations examined, the breeding period was short (generally less than two weeks) with the majority of breeding and oviposition activity confined to one or two nights. These observations are consistent with other studies that also found short breeding periods and explosive breeding activity in *B. boreas.* The length of breeding seasons for three large (>250 individuals) *B. boreas* populations in Oregon studied over five years ranged from 5–23 days, with the longer lengths attributed to prolonged unfavorable weather (Olson, 1988; Olson et al., 1986). Similarly, reported breeding periods for a large population of *B. canorus* are only slightly longer at 16–30 days (Kagarise Sherman, 1980). At the three *B. canorus* populations studied here, which were all much smaller than the population examined by Kagarise Sherman (1980), the majority of breeding activity was confined to only a few days. Although more intensive studies would be useful, duration of breeding period does not

appear to differ dramatically between the calling and non-calling populations of *B. boreas.*

Densities also did not appear to differ dramatically among sites. However, historical densities may have differed from current densities. For example, land use patterns in Alberta have dramatically altered *B. boreas* habitat. Most of the toad breeding localities examined for this study were man-made ponds and densities may be different at these sites than in more traditional habitat. More formal assessments of toad densities at breeding sites across the range of this species would be useful for further examining this hypothesis.

Another possibility is that natural selection pressures on calling males differ dramatically across the range of this species (sensu Zuk et al., 2006). Although anuran advertisement calls are intended as signals to conspecifics, other organisms may hear the call or otherwise observe calling males (Zuk and Kolluru, 1998, and references therein; Bernal et al., 2007). The calling populations occur east of the Continental Divide. This biogeographic pattern suggests that the calling populations may encounter a different suite of predators and parasites than their non-calling conspecifics west of the Continental Divide. If the threat from eavesdropping predators and parasites is greater west of the Continental Divide, then this could explain the loss of the call in these populations. Studies investigating potential predator and parasite responses to calling toads would help to address whether there are important geographic patterns in predation and parasitism.

Correlated changes in mate acquisition strategies

Wells (1977) noted dramatic differences between the mate acquisition strategies of explosive (high density) and prolonged (low density) breeders. Further, he noted that many species do not fit into just one class but may change strategies depending on the density of the chorus. At low densities, males call, but as the chorus density increases more and more males employ active searching. Density-dependent changes in mate acquisition strategies are known in *B. canorus* (Kagarise Sherman, 1980). At higher densities, males call less and begin actively searching for mates. While searching, males regularly attempt amplexus with conspecific males, resulting in numerous release calls being emitted from such aggregations of toads. In the *B. canorus* studied here, males routinely called from one area but then left to pursue any movement within a several meter radius. Subsequently, males often returned to the general vicinity where they started and resumed calling activity. These males clearly used both active searching and calling strategies.

Although males in some populations of *B. boreas* produce long, pulsed calls, these males do not entirely rely on calling for mate acquisition. As with their non-calling conspecifics and *B. canorus,* they continue to employ a mix of active searching and calling. In the calling and non-calling populations of *B. boreas*, males actively searched for females, although they generally patrolled only portions of a breeding site as opposed to the entire breeding area. More detailed behavioral studies of the calling populations would be useful for assessing whether there are correlations between call production and chorus density as was observed for *B. canorus* (Kagarise Sherman, 1980). Additional

studies of time spent searching and area or distance searched across populations with different call types would also be informative.

Is the Montana "advertisement" call intermediate between release calls and the Alberta advertisement call?

Interestingly, the lower-amplitude, long, pulsed call appears to occur in an area (Population 5) that is geographically intermediate between the non-calling populations from the majority of the range and the advertisement calling populations largely restricted to Alberta. Just as it is geographically intermediate, this call is in many ways intermediate between the release calls and the high-amplitude, long pulsed calls of the northeastern populations. Acoustically, this intermediate call is in several ways an extra-long release call. In *B. canorus* and the Alberta *B. boreas* populations, the dominant frequency of each individual's advertisement calls is almost always greater than the DF of the release calls (*B. canorus:* mean difference in DF between call types = 161.2 Hz, range = -100.7–287.7 Hz, n = 13; Alberta *B. boreas:* mean DF = 236.85 Hz, range = 121.7–302.9 Hz, n = 9). However, in the three Montana individuals for which both release and "advertisement" calls are available, the "advertisement" calls have a slightly lower dominant frequency (mean = -49.4 Hz, range = -3.7– -79.4 Hz, n = 3). The "advertisement" calls in the Montana population differ from typical advertisement calls because there is no initial transient for frequency; instead, the initial pulses have a slightly higher DF than later pulses. The measurement of dominant frequency of the "advertisement" calls, however, is from the middle of the call. Therefore, DF of the initial pulses of the "advertisement" call

is even more similar to the dominant frequencies of the release call pulses. These observations of DF for advertisement and release calls also are counter to Martin's (1971) claim that dominant frequencies of advertisement and release calls of the same individual toads are equivalent.

Patterns of amplitude variation within and between pulses also suggest that the "advertisement" calls of the Montana population are extended release calls. For *B. canorus* and the Alberta *B. boreas* the individual pulses in the release and advertisement calls have different patterns of interior amplitude modulation (Fig. 2.4). In particular, in the Alberta *B. boreas,* the release calls have a Type II pattern of amplitude modulation with numerous sub-pulses while the advertisement call pulses typically are Type III and lack sub-pulses (Table 2.4; Fig. 2.4). However, in the Montana population, the individual pulses of the "advertisement" and release calls show very similar Type II amplitude modulation (Table 2.4; Fig. 2.4). Finally, the variation in amplitude of subsequent pulses is much greater in the "advertisement" calls of Montana populations. These values are more similar to those found in release calls than in the other advertisement calls (Table 2.4).

The initial pulses of both the "advertisement" calls and release calls are also very similar. Release calls in toads consist of two components, a release vibration and a release chirp (Aronson, 1944; Brown and Littlejohn, 1972). The vibrations are produced by both males and females while the chirp, which is the audible pulse, is produced only by males. In a typical release call sequence, the vibration begins first with contraction of the thoracic musculature but without any simultaneous chirp production. In later pulses,

sound is also produced with a pulse of sound accompanying each muscular contraction. The "advertisement" calls begin similarly with contraction of the thoracic musculature producing pulses that either lack sound or pulses at an amplitude indistinguishable from the background (Fig. 2.4). This pattern is distinctly different from the advertisement calls in the Alberta *B. boreas* in which initial pulses are audible and in most cases distinguishable from the background.

There are also similarities between the Montana "advertisement" calls and the advertisement calls of *B. canorus* and Alberta *B. boreas.* Call duration, pulse number, and interpulse interval of the Montana "advertisement" call are all more similar to the values reported for the other advertisement calls (Table 2.4). Behaviorally, the calls are also given in a similar manner to typical advertisement calls. At present, "advertisement" and release calls from only three individuals from the Montana population were available for analysis. Further examination of this hypothesis will require examining calls of additional individuals and ideally additional populations with this unique call type.

Models of mating signal evolution

This work is the first to demonstrate among-population variation in the presence/absence of the major mating signal. The rapid evolution of the mating signal makes *B. boreas* ideal for numerous studies of sexual selection and mating signal evolution. In particular, documenting the occurrence of the advertisement call is one component of testing the underlying models of female preference evolution. These models are divided into two main categories, direct and indirect. One common way to

differentiate between these two classes of models is to assess whether the male trait and female preference are evolving in concert (Kirkpatrick and Ryan, 1991; Ryan and Rand, 1993; Morris et al., 1996). Indirect models (such as runaway and good genes) require that the female preference and the male trait are evolving in concert. Therefore, females in the non-calling populations that make up most of *B. boreas'* range should not have a preference for the loud long call, but females in the Alberta populations should. If such a pattern is not found, then direct models are favored. Work is currently underway to assess female preferences in multiple populations across the range of this species.

TABLE 2.1. Localities and numbers of individuals, advertisement calls (AC), and release calls (RC) analyzed per site for *Bufo boreas* and *Bufo canorus.*

Population	Date Recorded	No. of males	No. of AC	No. of RC
Bufo boreas				
1. Along Hwy 881, vicinity of Imperial Mills, Alberta, Canada. 55.0056°N, 111.73901°W	13–15-June-2004	5	25	25
2. University of Alberta, Meanook Biological Research Station (and vicinity), ca. 12 km SW of Athabasca, Alberta, Canada. 54.615°N, 113.343°W	17–20-June-2004 and 15-May to 1-June-2005	5	25	25
3. Ponds, ca. 4km NW of west entrance gate to Elk Island National Park, ca. 40km NE of Edmonton, Alberta, Canada. 53.704°N, 112.928°W	26-May to 1-June-2004	5	22	0
4. Vicinity of Lodgepole Alberta, Canada. Site 1, ca. 9 km ESE of Lodgepole, 53.06668°N, 115.19076°W. Site 2, ca. 2 km SE of Lodgepole, 53.09087°N, 115.30561°W.	2–5-June-2004	5	25	0
5. Lost Trail National Wildlife Refuge, north of Marion, Montana, USA.	16-May-2006 and 13-May-2007	3	15	15
Bufo canorus				
1. Sardine Meadow, 2.1 rd. km east of Sonora Pass on Hwy 108, Mono Co., California, USA. 38.32812°N, 119.61736°W	18–23-May-2004	8	37	38
2. Meadow on the southwest side of Lake Mary, Mono Co., California, USA. 37.60075°N, 119.00433°W	21–22-May-2004	5	25	21

TABLE 2.2. List of call characters examined. Some characters were measured differently for advertisement calls (AC) and release calls (RC).

Call Character	Description
Call rate	(Total number of calls -1) / time from beginning of first call to beginning of last call. Measured for AC only.
Dominant frequency	Frequency (Hz) in call containing the greatest energy.
Call duration	Time from beginning to end of one call (ms).
Pulse number	Total number of pulses in call.
Pulse rate	AC: (total number of pulses – 1) / time from beginning of first pulse to beginning of last pulse (s). Taken from 10 pulses from mid-call. RC: (total number of pulses – 1) / time from end of first pulse to end of last pulse.
Pulse duration	Time from beginning to end of one pulse (ms). AC: Average from 10 pulses from mid-call. RC: Average of all pulses except first pulse.
Interpulse interval	Time from end of one pulse to beginning of next pulse (ms). AC: Average from 10 pulses from mid-call. RC: Average of intervals following all pulses except the first and last pulses.
Pulse rise time	Time from beginning of pulse to point of maximum amplitude. AC: Average from 10 pulses from mid-call. RC: Average of all pulses except first pulse.
Pulse shape	Pulse rise time / pulse length (unitless variable). AC: Average from 10 pulses from mid-call. RC: Average of all pulses except first pulse.
Pulse duty cycle	Pulse duration / interpulse interval (unitless variable). AC: Average from 10 pulses from mid-call. RC: Average of all pulses except first pulse.
Call rise time	Time from call onset to first pulse with ≥ 75% call maximum amplitude. Measured for AC only.
Frequency modulation	The lowest dominant frequency from a pulse in the mid-call transect minus dominant frequency of first pulse of call (excluding any high frequency initial pulses[1]). Measured for AC only.
Amplitude Variation	Average difference in normalized amplitude among subsequent pulses. AC: Average from 10 pulses from mid-call. RC: Average of all pulses except first pulse.
Pulse type	Pulses with or without interior amplitude modulation.

[1]High frequency initial pulses are pulse(s) at the beginning of the call with a dominant frequency greater than the dominant frequency of any other pulses in the call.

TABLE 2.3. Regression equations for call characters significantly correlated with temperature.

Character	Regression Line	n	r	*P*
B. boreas Advertisement Call (9.0–19.4°C)		20		
Dominant Frequency (Hz)	y = 15.03x + 1147.80	20	0.484	0.031
Call Duration (ms)	y = -810.40x + 23259.86	20	0.734	<0.001
Pulse Rate (pulses/s)	y = 0.40x + 1.41	19	0.896	<0.001
Pulse Duration (ms)	y = -2.42x + 89.07	20	0.763	<0.001
Interpulse Interval	y = -6.69x + 188.68	18	0.929	<0.001
Pulse Rise Time (ms)	y = -1.25x + 42.61	20	0.690	0.001
Call Rise Time	y = -195.39x + 4339.25	19	0.859	<0.001
B. canorus Advertisement Call (17.0–24.6°C)		13		
Pulse Rate (pulses/s)	y = 0.57x + 1.57	11	0.908	<0.001
Pulse Duration (ms)	y = -1.42x + 63.55	12	0.920	<0.001
Interpulse Interval	y = -1.63x + 75.90	13	0.656	0.015
B. boreas Release Call (9.8–20°C)		10		
Pulse Number	y = 0.39x + 1.18	10	0.735	0.016
Pulse Rate (pulses/s)	y = 0.34x -0.035	10	0.986	<0.001
Pulse Duration (ms)	y = -2.79x + 95.37	9	0.868	0.002
Interpulse Interval (ms)	y = -12.81x + 347.81	10	0.935	<0.001
B. canorus Release Call (17.0–24.6°C)		13		
Pulse Duration (ms)	y = -1.14x + 53.27	13	0.605	0.028

TABLE 2.4. Summary values for measured call variables. For characters correlated with temperature (see Table 2.1), values are reported at 18°C[a]. The top row of each cell is the mean of the individual averages and the bottom row is the range of the individual averages.

	SVL (mm)	Call Rate (calls/min)	Dominant Frequency (Hz)	Call Duration (s)	No. of Pulses	Pulse Rate (pulses/s)	Pulse Duration (ms)	Interpulse interval (ms)
B. canorus	59.31	4.95	1393.08	4359.21	60.67	11.93	37.15	39.84
advertisement call	53–66	2.04–7.96	1142.12–1673.56	2609.68–9239.64[c]	39.6–105.4	9.87–14.53	29.46–40.17	31.48–51.59
B. boreas (Alb.)	68.0	2.63	1418.42	8672.6	81.33	8.73	45.51	86.63
advertisement call	60–74	1.31–4.72	1283.8–1562.9	5254.7–13303.5	58–113	7.64–10.87	34.17–55.33	58.76–133.85
B. boreas (MT)	91.0	2.78	924.53	7695.37	54.7	7.72	38.07	96.34
advertisement call[d]	86–95	2.11–3.88	(888.24–947.58)	(5679.0–10716.6)	44.2–65.2	(5.96–9.02)	(31.16–42.14)	(80.75–127.44)
B. canorus	58.15	N/A	1231.87	604.79	6.34	9.51	32.74	81.06
release call	53–66		1089.58–1378.99	330.23–1071.85	4.0–10.8	7.07–12.91	25.60–39.55	53.73–118.94
B. boreas (Alb.)	67.1	N/A	1176.16	1263.90	8.25	6.09	47.54	117.15
release call	60–74		1014.39–1292.21	796.88–1971.50	6.2–10.0	5.72–6.36	37.01–68.16	83.71–140.06
B. boreas (MT)	91.0	N/A	973.95	1786.05	11.4	6.02	61.63	114.09
release call[d]	86–95		(951.25–1017.16)	(1606.8–2133.1)	7.2–14	(4.12–7.66)	(53.76–73.61)	(74.71–169.66)

	Pulse Rise Time (ms)	Pulse Shape	Pulse Duty Cycle	Call Rise Time (s)	Frequency Modulation (Hz)	Amplitude Variation	Interior Amp. Modulation
B. canorus	11.66	0.371	0.807	741.40	63.77	0.072	Yes
advertisement call	8.37–18.08	0.269–0.483	0.603–0.963	335.10–1505.17	0–163.65	0.032–0.145	Type II
B. boreas (Alb.)	20.06	0.448	0.633	750.56	96.93	0.052	No–Minimal
advertisement call	12.73–26.77	0.401–0.505	0.472–0.872	0–1454.37[b]	30.15–180.88	0.017–0.152	Type III
B. boreas (MT)	15.54	0.408	0.416	N/A	N/A	0.141	Yes
advertisement call[d]	(11.86–21.82)	(0.290–0.522)	(0.322–0.531)			0.104–0.172	Type II
B. canorus	4.80	0.168	0.384	N/A	N/A	0.126	Yes
release call	1.45–7.71	0.068–0.296	0.234–0.635			0.074–0.207	Type II
B. boreas (Alb.)	10.71	0.185	0.370	N/A	N/A	0.110	Yes
release call	1.92–27.51	0.032–0.322	0.281–0.483			0.044–0.188	Type II
B. boreas (MT)	20.81	0.350	0.608	N/A	N/A	0.90	Yes
release call[d]	(16.94–27.65)	(0.230–0.516)	(0.452–0.737)			0.074–0.110	Type II

[a]Values for the Montana *B. boreas* were not evaluated for correlations with temperature because only three animals were available for analysis and their mean call temperature (18.4°C) was very similar to the standardized value.
[b] A negative value was obtained for one individual following temperature correction to 18°C so a 0 is reported for the minimum call rise time. However, the minimum call rise time observed prior to temperature correction was 548.63 ms.

[c] One *B. canorus* gave unusually long calls; of the 57 calls examined from the remaining 12 individuals, the longest call was 7226.37 ms. For each of these 12 individuals, the average call durations are 2609.68–5089.72 ms.

[d] In the Montana *B. boreas,* parentheses around ranges denote those characters that are likely to vary with temperature. Because not enough individuals were available to determine the regression equation (or whether there is a significant relationship), these uncorrected measurements reflect both intrapopulation variation and variation due to differences in calling temperature (see Methods).

TABLE 2.5. Number of males of various taxa with and without vocal sacs.

Taxon	No. males without vocal sacs	No. males with vocal sacs	No. of males with two vocal slits
Bufo boreas	953	124	35
Bufo canorus	0	172	44
Bufo exsul	15	0	N/A
Bufo nelsoni	15	0	N/A
B. boreas x *B. punctatus*	0	2	1
B. boreas x *B. canorus*	3	10	6

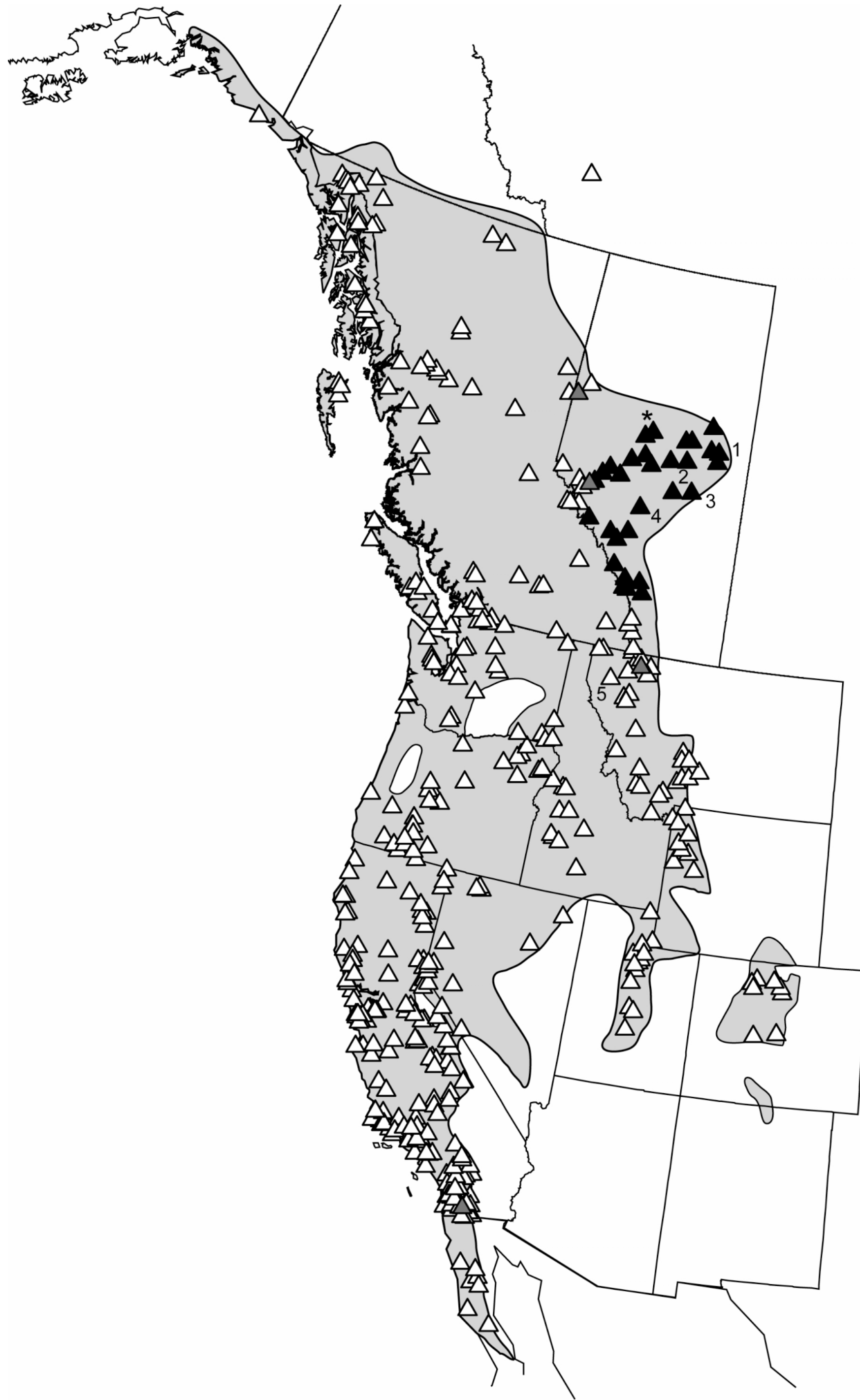
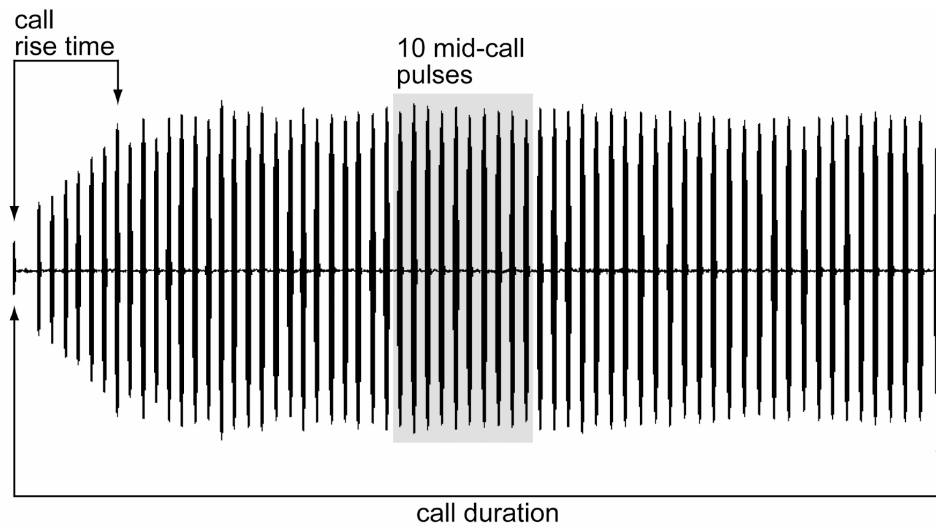


FIGURE 2.1. Range map of *Bufo boreas* (modified from Stebbins, 1985) with localities of museum specimens examined for the occurrence of vocal sacs depicted (n = 419). At each site, 1–31 males were examined, and either all males lacked vocal sacs (white), all males had vocal sacs (black), or males with and without vocal sacs were present (gray). Numbered sites in Alberta are localities where males were recorded. The asterisk in northern Alberta is a site where individuals were observed to have vocal sacs and produce long, pulsed calls but no specimens or recordings were taken.



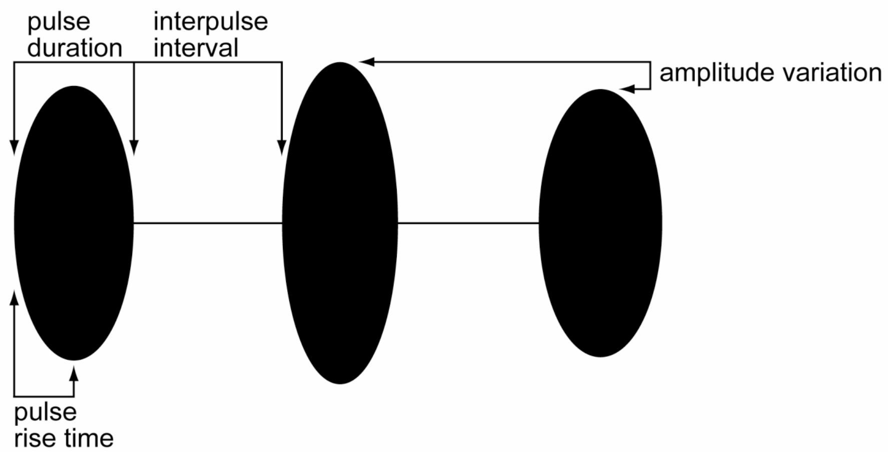


FIGURE 2.2. Waveforms (amplitude through time) of a *Bufo boreas* advertisement call and three schematic pulses depicting call variables measured for this study.

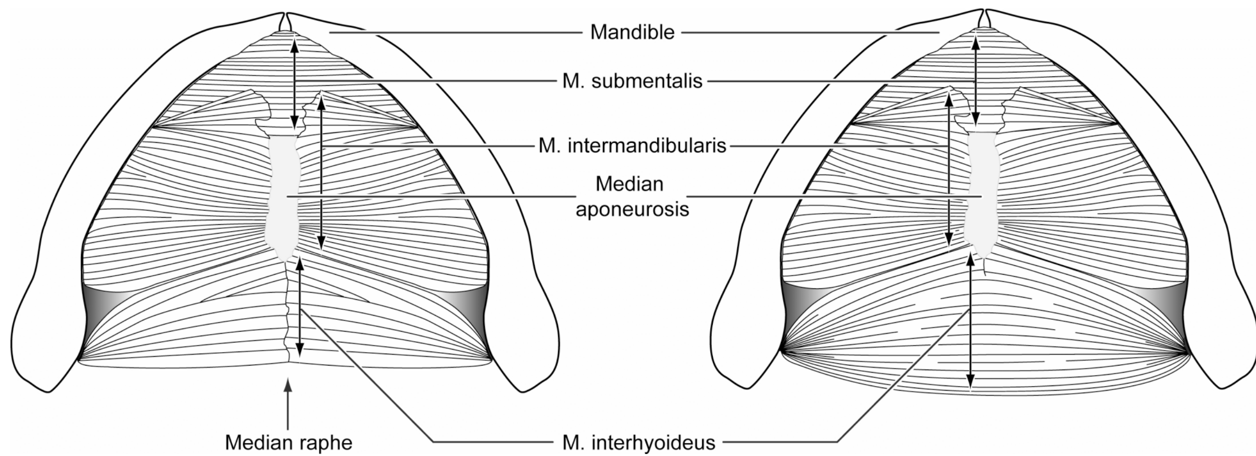


FIGURE 2.3. Intermandibular structures of *B. boreas* without (left) and with (right) vocal sacs. Individuals lacking vocal sacs include some adult males, all adult females, and all sub-adults. In the development of the vocal sac, vocal slits form in the buccal cavity, the median raphae recedes anteriorly, the muscle fibers of the interhyoideus become long, extending across the midline, the muscle fibers become more widely spaced with the development of connective tissue between parallel fibers, and the interhyoideus shows development posteriorly and takes on a pouch-like appearance as opposed to being a flat sheet of muscle.

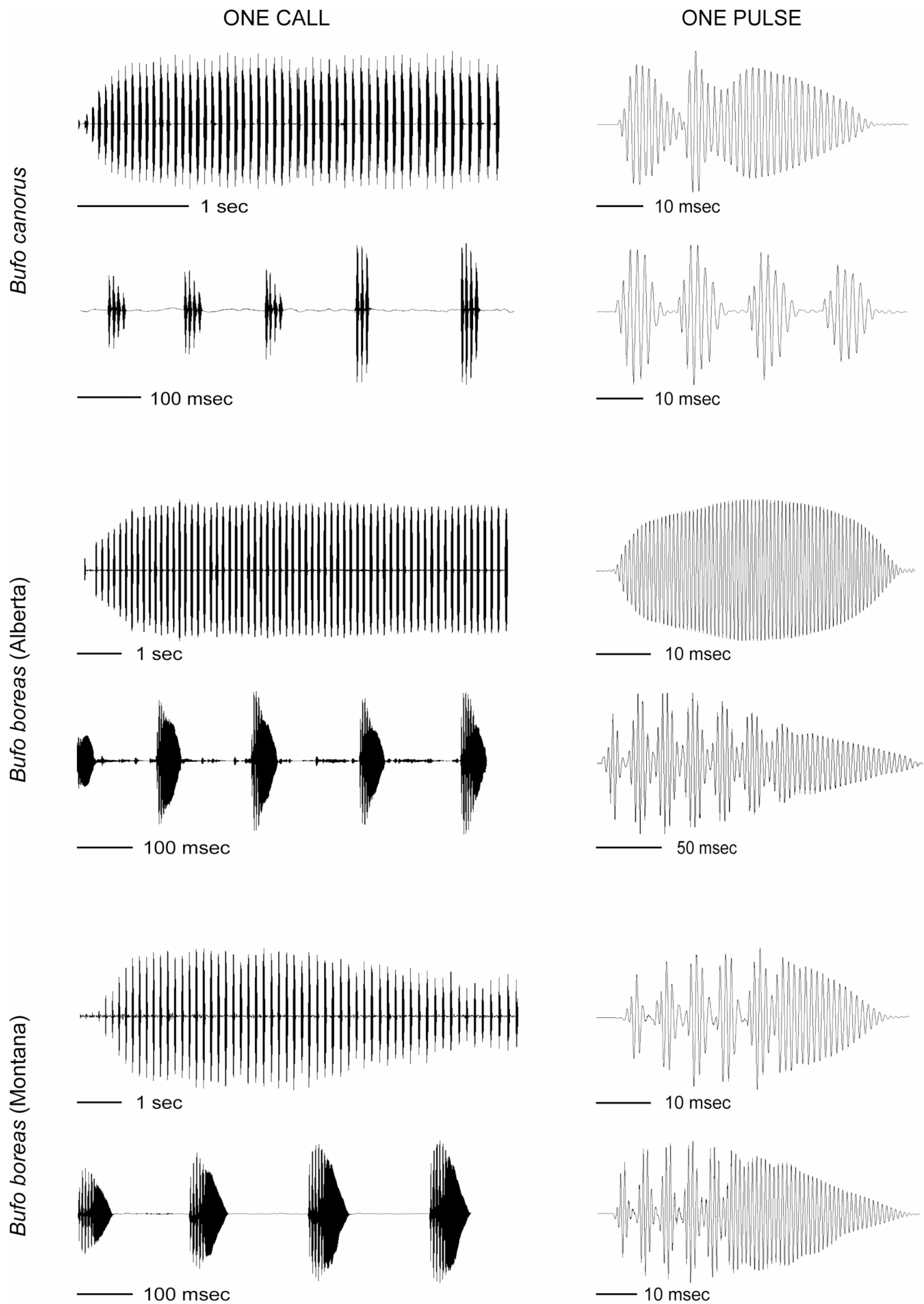


FIGURE 2.4. Representative waveforms (amplitude through time) of advertisement (top) and release (bottom) calls of *B. canorus, B. boreas* with vocal sacs (Population 2 from Alberta), and *B. boreas* without vocal sacs (Population 5 from Montana). The left column is the waveform of an entire call and the right column is a waveform of a single pulse from that call.

Chapter 3: Sensory Exploitation and the Re-evolution of the Primary Mating Signal in Western Toads, *Bufo boreas*

3.1 Introduction

The long, colorful plumes of peacocks, the visual and acoustic displays of sage-grouse, and the calls of many songbirds, anurans, and insects exemplify the diverse morphologies and behaviors used by males of many species to attract mates. Although there are obvious disadvantages in terms of natural selection for such conspicuous displays, the increased mating success of displaying males (i.e., sexual selection by female choice) explains why such traits evolve. It is often less clear, however, why females have preferences for these exaggerated traits in the first place. Much recent work has suggested that female preferences can reflect preexisting biases in the female's sensory or cognitive systems to a particular signal or aspect of a signal (Ryan, 1990, 1998; Kirkpatrick and Ryan, 1991; Ryan and Rand, 1993; Basolo, 1995; Shaw, 1995; Endler and Basolo, 1998; Kokko et al., 2003). Typically, these biases are thought to result from evolution of the sensory system involved in mate choice but for reasons not related to the male signaling trait (e.g., for prey localization, predator avoidance, or for a different mate choice preference such as species recognition; Ryan, 1998). Male signals then evolve to exploit these pre-existing biases. For this reason, hypotheses describing such a scenario are termed sensory exploitation hypotheses.

Three criteria are generally considered necessary to demonstrate sensory exploitation (Basolo, 1995). 1) In the lineage in which the males have the display trait, females have a preference for the trait; 2) In closely related lineages in which males lack the display trait, females have the preference for the trait; 3) Absence of the male trait is the ancestral state in the clade of interest. Thus, identifying sensory exploitation requires first identifying clades in which there is variation in the presence of the signaling trait and then testing for the occurrence of the female preference in the displaying taxon and in closely related non-displaying taxa. Phylogenetic analyses are then used to determine whether the preference existed prior to the trait. A classic example of sensory exploitation is the fish genus *Xiphophorus,* in which males of some species have an extension of the caudal fin, termed a sword, while males of other species are swordless. Importantly, the preference for swords is an ancestral trait in the clade suggesting that the sword evolved to exploit this preexisting preference (Basolo, 1990, 1995).

Recent work has demonstrated variation in the presence of the major mating signal, the advertisement call, in the Western Toad, *Bufo boreas* (Pauly, 2008). Unlike in other taxa previously studied (e.g., swords in *Xiphophorus*), however, this variation occurs among populations of a single species and not among species of a larger clade. In *B. boreas,* only males from populations in the northeastern corner of its range produce the long, high-amplitude advertisement calls common to most *Bufo* (Pauly, 2008; Fig. 3.1). Further, it is only these calling males that have a vocal sac, which is a distensible balloon-like structure that expands with pulmonary air during calling. Males throughout the rest of the range do not produce this call and lack vocal sacs. (In at least one population in

Montana [Population 19, Fig. 3.1], males lacking vocal sacs do produce a long, trilled call, but this call is of low-amplitude, of unknown function, and acoustically different form the known advertisement call in northeastern populations. Thus, it is not considered further here.).

All Nearctic *Bufo* outside of the *B. boreas* species group produce advertisement calls, suggesting that the ancestor of the *B. boreas* clade also produced calls. Therefore, if the calling populations of *B. boreas* are the basal most lineages in this species, then call production was lost once resulting in the lack of calling in the majority of populations. Alternatively, if the calling populations are phylogenetically nested among non-calling lineages, then calling was likely re-evolved from a non-calling ancestor assuming that the transition call⇔no call has symmetric and equal probability. The re-evolution of the call would be consistent with a sensory exploitation hypothesis if the female preference for calls remained in the non-calling *B. boreas* ancestor to the calling populations.

Here, I test for the presence of the female preference in multiple non-calling populations and conduct DNA sequencing and phylogenetic analyses to assess the evolution of the male advertisement call and the female preference for it. Nuclear DNA analyses are also conducted to test whether the calling *B. boreas* populations are hybrids with the Canadian Toad, *Bufo hemiophrys.* These two species have a narrow zone of overlap in central and eastern Alberta, and can co-occur at breeding sites (Cook, 1983; Eaton et al., 1999). As with all other members of the *B. americanus* species group, *B. hemiophrys* has a vocal sac and produces advertisement calls. Hybridization between *B. boreas* at sites where males lack vocal sacs and other species of Nearctic *Bufo* (*Bufo*

punctatus and *Bufo canorus*) that have vocal sacs and call results in offspring with vocal sacs that presumably can call (Pauly, 2008). Although *B. boreas* and *B. hemiophrys* are not closely related (Pauly et al., 2004), a field-collected morphologically identified hybrid has been reported, although it is not known if it was fertile (Cook, 1983). Additionally, at least some hybrids from reciprocal laboratory crosses between *B. boreas* and *B. hemiophrys* reached metamorphosis (Blair, 1964b, 1972), suggesting that the appearance of vocal sacs and call production in the northeastern *B. boreas* could be due to hybridization.

3.2 METHODS

Pauly (2008) demonstrated the occurrence of calling and non-calling populations across the range of *B. boreas.* Only those populations in the northeastern portion of the range (Alberta and northern Montana) produce long, high-amplitude advertisement calls (Fig. 3.1). Further, phonotaxis tests demonstrated that females from a calling population in Alberta are able to recognize and respond to male advertisement calls (Pauly, 2008). Therefore, Criterion 1 for sensory exploitation has been met as females from the calling population have the preference for the call. To assess the evolutionary history of the call and preference it is necessary (1) to generate a phylogeny for the relevant populations and (2) to test for the female preference in non-calling populations.

Molecular Analyses

Two different DNA sequence analyses were conducted. The first used a rapidly evolving mtDNA marker to examine intraspecific relationships in *B. boreas.* The resulting phylogeny was used to assess the evolutionary history of the female preference and the male trait. The second analysis used nuclear gene sequences to test whether the appearance of vocal sacs and advertisement calling in the northeastern *B. boreas* populations results from hybridization between *B. boreas* and *B. hemiophrys.*

DNA was extracted using the Viogene DNA/RNA Extraction Kit. For the mtDNA study, approximately 930 nt of cytochrome b were amplified using CytbAR-H (Goebel et al., 1999) and a slightly modified version of the primer MVZ43 (Graybeal, 1993; 5'-GAGTCTGCCTAATTGCYCAAA-3') with the following thermal cycle profile: 2 min at 94ºC, followed by 35 cycles of 94ºC for 30 sec, 48ºC for 40 sec, and 72ºC for 90 sec, and a final extension phase at 72ºC for 7 minutes. This fragment was chosen because in *B. boreas* it was found to be the most rapidly evolving among several commonly sequenced rapidly evolving markers, including the D-loop (control region) and ND1. Purified PCR products were sequenced in both directions and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequences were edited and assembled using Sequencher 4.1 (Gene Codes Corp.) and manually aligned in MacClade 4.08 (Maddison and Maddison, 2000). Variable sites were verified by examining the original chromatograms.

Sampling for the mtDNA analysis included 42 *B. boreas* from throughout the species range (Appendix C). Each sample was from a different site. *Bufo americanus*

(TNHC 62701) and *B. punctatus* (TNHC 58788) were designated as outgroups based on Pauly et al. (2004).

Phylogenetic relationships were assessed using neighbor-joining (NJ) and maximum parsimony (MP) analyses in PAUP* (version 4.0b10; Swofford, 2003) and maximum likelihood (ML) analyses in GARLI (Zwickl, 2006). Several haplotypes were recovered in multiple individuals; a maximum of two representatives of each haplotype were included in the analyses. The NJ analysis consisted of a bootstrap with 1000 pseudoreplicates using uncorrected p-distances. To find the most-parsimonious tree, a heuristic search with 1000 random addition-sequence replicates and tree-bisection-reconnection (TBR) branch swapping was conducted. Nodal support was assessed through a non-parametric bootstrap analysis consisting of 1000 pseudoreplicates with 100 random addition-sequence replicates per bootstrap pseudoreplicate and TBR branch swapping. For the ML analysis, the most appropriate model of evolution was selected by examining the fit of successively more complex models using the Akaike Information Criterion (AIC) as implemented in MrModeltest (version 2.2; Nylander, 2004). The ML analysis consisted of a bootstrap with 1000 pseudoreplicates.

Bayesian analyses were also conducted to specifically test whether the calling populations are the basal-most lineages in *B. boreas.* This relationship would indicate that calling is the ancestral condition in *B. boreas* and was lost once resulting in the lack of calling in most populations. Bayesian analyses were conducted in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) using four runs of four chains each. The analysis used default priors with the model of evolution determined from the MrModeltest run

described above. Sampling frequency was set to once every 1000 generations. Convergence was determined using MrConverge v1.b.2 (a Java program written by A. R. Lemmon), which assesses convergence by examining stationarity of likelihood scores in each run and the precision of the bipartition posterior probability estimate from comparisons across the four runs (Brown and Lemmon, 2007).

For the hybridization study, the primers Rhod1U (modified from Hoegg et al., 2004; 5'-AACGGAACAGAAGGCCCAAACTT-3') and Rhod1L (developed by A. K. Holloway and D. C. Cannatella; 5'-GCCAAAGCCATGATCCAGGTGA-3') were used to amplify approximately 755 nt of rhodopsin using the same thermal cycle profile as used for the cytochrome b fragment except the annealing temperature was 55ºC. This fragment includes parts of rhodopsin exon 1 and exon 2 and the complete intervening intron. The sequencing and alignment protocols for this fragment were identical to that used for cytochrome b, except that some sequences were only sequenced in one direction as described below.

The goal of the nuclear gene sequencing was to determine whether the northeastern *B. boreas* populations are *B. boreas* x *B. hemiophrys* hybrids. Therefore, individuals of each parental species from outside of the potential area of hybridization were sequenced as well as a single *B. americanus,* a species closely related to *B. hemiophrys* (Pauly et al., 2004). This sample included 11 *B. boreas* from throughout this species' range and 5 *B. hemiophrys* (Appendix C). These sequences were used to determine the number of SNPs that differentiate *hemiophrys* and *boreas* alleles. Then, an additional 29 individuals from calling *B. boreas* populations in Alberta (i.e., potential

hybrids) were sequenced (Appendix C), and the SNP sites were examined to determine allele assignment. Because it is straightforward to differentiate among the two species based on sequence variation, 19 of the 29 Alberta sequences were sequenced in only one direction, which provided data for all 710 nucleotides internal to the primers.

Phonotaxis Testing

Phonotaxis tests were conducted at three sites where males do not produce the high amplitude, pulsed call found in the northeastern populations. These three sites were chosen to represent the major clades of *B. boreas* recovered in the phylogenetic analyses. The first locality was at Corral Hollow, which spans the Alameda-San Joaquin County Line in central California (Population 26). The second site was along the Sweetwater River in Descanso, San Diego County in southern California (Population 41). The third site was at Lost Trail National Wildlife Refuge in Flathead County, Montana (Population 19). This last site was selected because it is genetically close and geographically proximate to the calling populations in Montana and Alberta.

The testing protocol followed Pauly (2008), and additional details are provided there. Testing was conducted in a chamber (internal dimensions: 180 cm x 140 cm and ca. 100 cm tall) set up indoors in a completely darkened room to minimize light and auditory noise. Speakers were placed in the two corners of a long wall opposite each other and approximately 10 cm out from the wall allowing toads to move along the walls without contacting the speakers. A cone was placed at the midpoint of the long wall, opposite the wall with speakers. Female toads were captured at breeding ponds and

returned to the chamber for testing. Because male *B. boreas* actively search for females and will remain in amplexus with females for multiple days prior to breeding (Pauly, pers. obs.), finding females in amplexus does not necessarily mean that they are motivated to breed that night. Therefore, all females encountered at breeding sites were captured for testing regardless of whether or not they were in amplexus (although non-amplectant females were rarely found). For testing, a female was removed from amplexus (if necessary) and placed under the cone in the testing chamber. Test stimuli were then broadcast for three minutes at which time the cone was raised allowing the female to move about the chamber. The trial was filmed under infrared light and viewed on a video screen in an adjacent room. A positive phonotactic response was counted if a female approached to within 10 cm of a speaker front. Approaches to the side or back of the speaker were not counted unless the female moved to the front of the speaker. The trial was ended and no response was registered if the female did not move in the initial 5 minutes, did not move for 2 minutes after initial movements, did not choose within 15 minutes, or attempted to climb the chamber walls two times.

In all trials one speaker broadcast white noise (0–5000 Hz) and the other broadcast a hypothesized advertisement call from a local male. Each hypothesized advertisement call was made by selecting one call from a male from an Alberta population (Meanook Biological Research Station) and altering the dominant frequency of the call to reflect the average male body size in the test population. This was done because there is a significant negative correlation between dominant frequency and body size in this species (Pauly, 2008), and the adult male body sizes varied across

populations. The regression equation for dominant frequency against body size was determined from measurements of 16 males from the Meanook population ($y = -0.029x + 3.196$, $r = 0.608$, $P < 0.013$; Pauly, 2008). Average body sizes (snout-vent length, SVL) of males from the Corral Hollow (n = 21, mean = 81.21 mm, range = 69–94 mm), Sweetwater River (n = 21, mean = 77.86 mm, range = 63–90 mm), and Lost Trail (n = 50, mean = 91.28 mm, range = 80–103 mm) populations were then used for determining an appropriate frequency for the test calls. This approach assumes that the relationship between dominant frequency and body size for the Meanook males is applicable to these other *B. boreas* populations. This assumption is most tenuous for the Lost Trail population as the minimum SVL of an adult Lost Trail male (80 mm) was much greater than the largest males from Meanook (range = 59–67 mm). Using the mean body size of a Lost Trail male and the regression equation from the Meanook population results in a call with an extremely low dominant frequency (558 Hz). Therefore, calls set to a dominant frequency of 849 Hz were used in both the Corral Hollow and Lost Trail populations. Test calls were modified to the appropriate dominant frequency in CoolEdit Pro, version 2 (Syntrillium Software) using the pitch shift command.

Some calls were also modified in temporal characteristics to fit the testing environment. Several temporal components of calls vary with temperature. Therefore, when possible, the test call was selected so that the recording temperature and chamber temperature were within 1°C. If appropriate calls were not available for a given chamber temperature, the tempo of a call was modified in CoolEdit Pro using the time stretch

command according to the regression equation for pulse rate and temperature ($y = 0.534x - 1.162$, $r = 0.967$, $P < 0.001$; Pauly, 2008).

For testing, calls were broadcast at a call rate of 2.57 calls/minute, which is the average call rate of 10 individuals from the Meanook population. The white noise was set to be the same length as the call it was paired with and was broadcast antiphonally. The speaker broadcasting the call was assigned randomly. Once a female responded to a given call, that call was not used for further testing in that population. Stimuli were broadcast at 72 dB SPL (re 20 microPascals) at 50 cm, a value representative of the amplitudes of calls in Alberta populations (Pauly, 2008). Decibel level was checked prior to every test with a portable SPL meter (RadioShack Model No. 33-2055) set to C weighting and fast response.

3.3 RESULTS

Molecular Analyses

The final alignment was 866 nt long with 222 variable characters of which 93 were parsimony-informative. The maximum uncorrected percent sequence divergence in the ingroup was 4.04%. Within *B. boreas,* 22 distinct haplotypes were found. Nine most-parsimonious trees were recovered and only differ in relationships among very similar haplotypes. The haplotypes fall into three geographically defined groups: a southern group, a central group, and a northern group (Fig. 3.1). The southern and central groups were strongly supported in all analyses, but the monophyly of the northern group was

very low in the ML analysis. This low support largely reflects the placement of the root in many of the ML bootstraps. In the NJ and MP analyses, the placement of the root indicates that the central and northern clades are sister taxa (as depicted in Fig. 3.1; bootstrap support = 51 for NJ, and 53 for MP). An alternative arrangement that places the central and southern clades as sister taxa received slightly lower support (see "Alternative Root" in Fig. 3.1; bootstrap support = 49 for NJ, and 47 for MP). However, in the ML analyses, neither of these arrangements was frequently recovered. Instead, the root was typically placed such that one of several haplotypes in the northern group was recovered as the sister taxon to all other *B. boreas.* This results in the northern group being paraphyletic in these ML bootstrap pseudoreplicates. All reconstructions of the root in the ML analyses were very poorly supported.

The poor resolution of the root placement reflects the large distance between the ingroup and the two outgroups (15.2–17.8%). The *B. boreas* species group is the sister taxon to all other Nearctic *Bufo* (Pauly et al., 2004) so there are no other taxa outside of this species group that would serve as a more appropriate outgroup. The three remaining species in the *B. boreas* species group, *Bufo nelsoni, Bufo exsul,* and *Bufo canorus,* are also inappropriate choices for outgroups. Previous and ongoing research using nuclear and mitochondrial markers demonstrates that *B. nelsoni* and *B. exsul* are each nested within *B. boreas* (Feder, 1977; Graybeal, 1993; Goebel, 1996; Pauly et al., 2004; Pauly, unpubl. data). Additionally, although the Yosemite Toad, *B. canorus,* is the sister taxon to *B. boreas* based on nuclear data (re-analyses of Feder's [1977] allozyme dataset and nuclear sequencing of two genes; Pauly, unpubl. data), extensive mitochondrial

introgression from *B. boreas* into *B. canorus* makes it unsuitable for an outgroup (Pauly et al., 2004). Fortunately, two studies using slower evolving markers also have adequate sampling of multiple *B. boreas* and other toad species and indicate the appropriate placement of the root for *B. boreas*. Both Goebel (1996) and Pauly et al. (2004) placed the root along the same branch as found in the MP and NJ analyses, and this placement is treated as the most probable here. Further, both studies also recovered the three geographically defined clades.

Bayesian analyses were used to specifically test whether the calling populations are the most basal lineages of *B. boreas.* Convergence was found to have occurred by 18,000 generations so the first 18 trees of each of the four runs were discarded as the burn-in. The remaining trees were combined, yielding 3932 trees for determining Bayesian posterior probabilities. By using a filter with a constraint tree, only 2% of all post burn-in trees were found to be consistent with the calling haplotypes being the most basal lineages in *B. boreas.*

The phylogenetic results demonstrate that the calling populations share identical or nearly identical haplotypes. Assuming that the root is in one of the placements depicted in Fig. 3.1, then the calling populations are nested within a widely distributed clade of non-calling individuals (Fig. 3.1). Thus, advertisement calling and vocal sacs appear to have been lost in ancestral *B. boreas* and then secondarily re-evolved in the ancestor to the northeastern populations (Fig. 3.1).

For the 710 nt segment of rhodopsin, all *B. hemiophrys* sequences (and the single *B. americanus* sequence) differed from all *B. boreas* by 22 SNPs and two deletions. Thus,

it is uncomplicated to detect *B. hemiophrys* alleles if they are present in the calling *B. boreas.* Both deletions were in the intron and were 14 and 23 nucleotides long. All 29 individuals (i.e., 58 alleles) from the five calling Alberta *B. boreas* populations had alleles identical to those of other *B. boreas.* That is, for each of these 58 alleles, all had the *boreas*-specific nucleotide at SNP sites and lacked the two deletions observed in *B. hemiophrys* and *B. americanus.* Thus, the genetic evidence suggests no gene flow between *B. boreas* and *B. hemiophrys* in Alberta.

Phonotaxis Testing

In all populations tested, females approached the speaker broadcasting the call far more frequently than the speaker broadcasting noise (Table 3.1). Only one female was counted as having gone to the noise speaker across all four populations (i.e., the three non-calling populations examined here and the one calling population in Alberta examined by Pauly, 2008). In nearly all cases when a female approached the call speaker, she did so quickly and directly. A one-tailed binomial test was used to test the hypothesis that females go to the call speaker significantly more often than they go to the noise speaker. If females could not recognize and respond to the call, then approaches to the call and noise speakers should occur at equal frequencies. Significantly more approaches to the call speaker were found in the Alberta and central California populations (at alpha = 0.05). Although only small sample sizes were attained at the other two sites in Southern California and Montana, the trend was also towards the call speakers (Table 3.1). These

results indicate all female *B. boreas* have the preference for advertisement calls, regardless of whether or not males in their population produce them.

3.4 Discussion

Numerous models have been proposed to explain the evolution of female preferences for exaggerated display traits. These models do not necessarily explain the origin of the trait but how the preference and male trait evolve through time. These models can be classified into two categories depending on how selection influences the evolution of the preference (reviewed in Kirkpatrick and Ryan, 1991). Under direct selection models, there is selection directly on the female's preferences because her choice affects her own survival or fecundity. Under indirect selection models, such as "good genes" or Fisher's theory of runaway sexual selection, there is a genetic correlation between the male trait and female preference such that the preference evolves under indirect selection as a correlated response to evolution of the male trait, which is under direct selection by the female preference. Given that there is heritable variation for both the trait and the preference, this genetic correlation results from linkage disequilibrium simply because the females with the most extreme preference will on average mate with males with the most extreme trait. A straightforward way that has been used to test whether indirect or direct models are operating is to use phylogenetic analyses to determine whether the trait and preference have evolved in concert (Kirkpatrick and Ryan, 1991). In *B. boreas,* indirect models can be rejected because the advertisement call

and the preference for it clearly have not evolved in concert. Although the advertisement call is lost in some populations, the preference for it is not.

Interpreting whether 1) calling was ancestral in *B. boreas* and then lost or 2) non-calling was ancestral and calling re-evolved in the northeastern populations is in large part dependent upon the placement of the root. Unfortunately, placement of the root is complicated by the relatively large sequence divergence between the ingroup and potential outgroups. The latter scenario (re-evolution of calling) is favored by the NJ and MP analyses (Fig. 3.1), but the sequence of gains and/or losses is not clear in the likelihood analyses where there is no clear support for any placement of the root. In the likelihood and Bayesian analyses, all placements of the root, including the two common placements found in the NJ and MP analyses, receive less than 50% likelihood bootstrap and Bayesian posterior probability support. The Bayesian analysis does reject the hypothesis that the calling haplotypes are basal to the non-calling lineages so a single loss of calling can be rejected. Nevertheless, because the root is often recovered as being within the northern group in the likelihood analyses, multiple losses of calling could explain the observed occurrence of calling and non-calling lineages. Multiple studies using slower evolving markers more appropriate for estimating root placement for *B. boreas*, however, recover the northern group as monophyletic and place the root basal to this lineage in both model (ML) and non-model (NJ and MP) based analyses (Goebel, 1996; Pauly et al., 2004). Given that these studies agree with the root placement in the NJ and MP analyses and the root placement in some of the ML and Bayesian reconstructions

for the cytochrome b data, this placement and the monophyly of the northern lineage, is treated as most probable here.

Rooting such that the northern lineage is monophyletic (i.e., the favored rooting or the alternative rooting in Fig. 3.1) suggests that the long, high-amplitude advertisement calls and vocal sacs in northeastern populations of *B. boreas* evolved from an ancestor that lacked such an advertisement call or a vocal sac (Fig. 3.1). The Nearctic *Bufo* is one of three major lineages that makes up the New World Clade of *Bufo* (Pauly et al., 2004), which includes approximately 135 species. Nearly all of these species produce advertisement calls and have vocal sacs. Further, *B. canorus,* the sister species to *B. boreas,* also produces an advertisement call. Thus, the loss of calling appears to have occurred in the ancestral lineage to *B. boreas* with the lack of calling now widespread throughout its range (Fig. 3.1). More recently, calling and the presence of vocal sacs appear to have been re-evolved in the northeastern populations. It is likely that other morphological and behavioral traits, such as changes in larynx size and/or shape, are also associated with the return to advertisement calling, and this is being investigated currently.

The re-evolution of the call in the northeastern populations of *B. boreas* is consistent with sensory exploitation. Females have the preference for the call in populations where the male trait is present (Criterion 1) and absent (Criterion 2), and the call is absent in the ancestor to the calling populations (Criterion 3). Thus, in the northeastern populations, the call evolved to exploit a preexisting preference. Often, in sensory exploitation, the male trait is thought to be exploiting a preference that evolved

because it is adaptive in some context separate from mate acquisition (Kirkpatrick and Ryan, 1991; Enquist and Arak, 1993). For example, water mites use water-borne vibrations to detect their copepod prey, and, at least once, male water mites have evolved a leg-trembling behavior in their courtship display that mimics these vibrations apparently to increase attention from females (Proctor, 1991, 1992). Preexisting receiver biases, however, can originate from a variety of sources (Ryan, 1998). For the calling *B. boreas*, the male trait appears to be exploiting a preference that exists because of retention of the ancestral preference for the call. Although the call was lost, the preference for the call remained, establishing a bias that was later exploited with the re-evolution of the call.

TABLE 3.1. Results of phonotaxis tests in four populations of *Bufo boreas*. Males in the Alberta population produce a long, high-amplitude advertisement call while males in the other populations do not produce this call.

Population	No. approach call speaker	No. approach white noise speaker	*P*
Alberta (call)	24	0	<0.001
Central California	11	0	0.005
Southern California	6	1	0.547
Montana	2	0	0.25

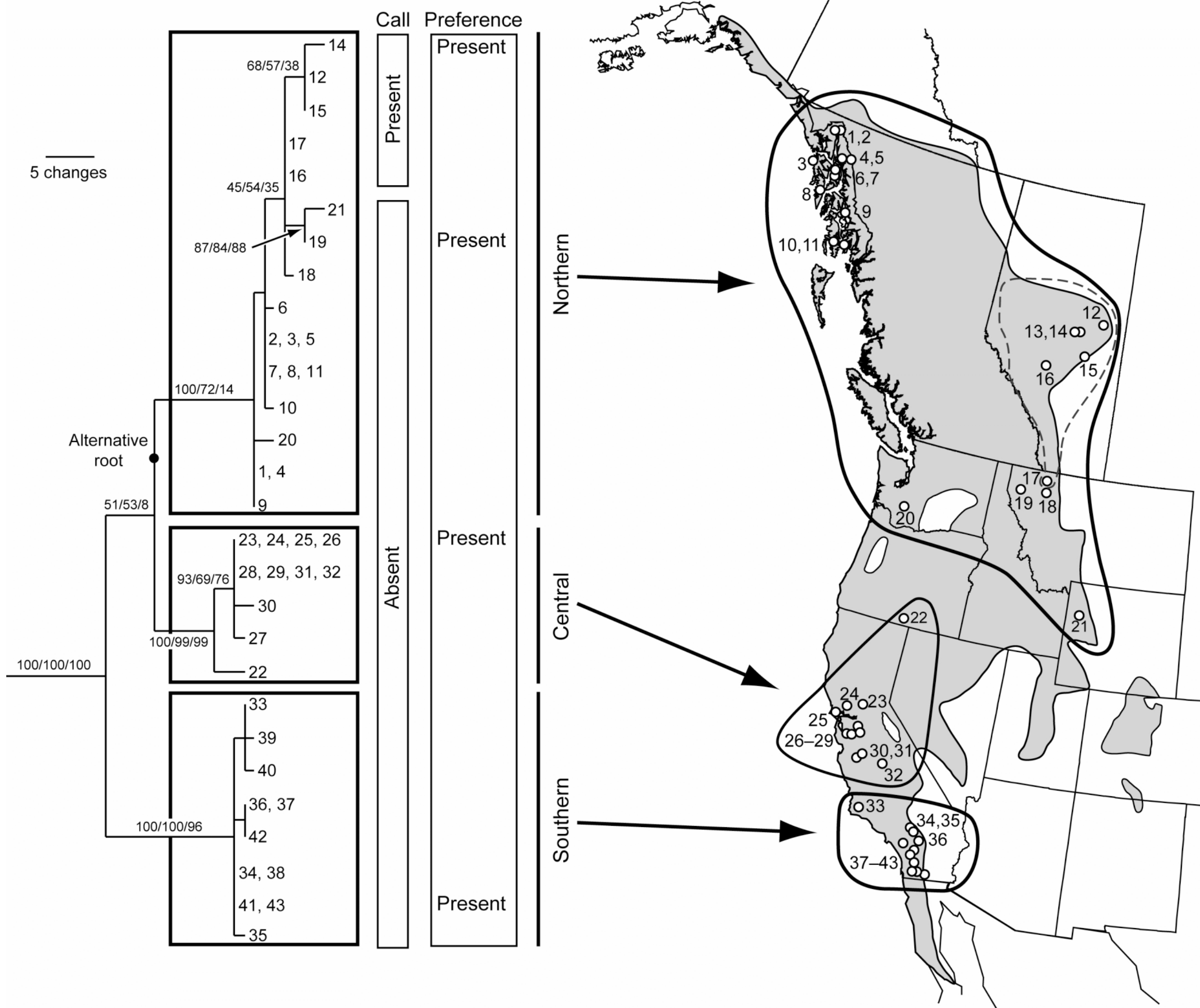


FIGURE 3.1. Range map of *Bufo boreas* and one of the nine most-parsimonious trees from analyses of 42 individuals. The shaded area depicts the range of *Bufo boreas* with the populations sampled in the molecular analyses shown; numbers correspond to Appendix C. The dashed dark gray line encircles that portion of *B. boreas'* range where males produce long, high-amplitude advertisement calls (Pauly, 2008). In the phylogram, an alternative placement for the root, as described in the Results, is also shown. Bootstrap support values are reported for the neighbor-joining/parsimony/likelihood analyses. Populations in the phylogram are labeled as to whether or not males produce the advertisement call (based on Pauly, 2008) and as to membership in each of the three main geographic clades. Lack of calling was determined by absence of the vocal sacs in museum specimens and, in some populations, from observations during the breeding season (see Pauly, 2008). Similarly, the presence of calling in population 17 in Montana is inferred from the presence of vocal sacs in museum specimens (Pauly, 2008). The presence of the female preference for this call is assumed to occur in all population because it is present in the four populations (14, 19, 26, and 41) in which testing was conducted (see Table 3.

Chapter 4: Sources and Impacts of Sequencing Error and a Novel Approach for Detecting Sequence Errors in rDNA

4.1 Introduction

Sequencing error is inherent to the generation of DNA sequence data. Errors can result from misincorporation of nucleotides during the polymerase chain reaction (PCR; either during standard amplification or sequencing reactions), misreads by the sequencing analysis software, editing errors by researchers, or from sequencing of vectors or contaminants. If unrecognized, these errors can have serious consequences for both the research projects for which the sequences were generated and for future studies if the data become available in a public database (e.g., GenBank). Moreover, when sequence data are submitted to public databases, the sequences are almost always separated from supporting information, such as quality scores and chromatograms, that indicate sequence accuracy and data quality. As a result, assessing anomalies and potential errors becomes more difficult for subsequent investigators.

Here, I briefly review sources of errors, estimates of sequencing errors in public databases, and the impact that these errors can have on biological research. I argue that sequencing errors can dramatically impact study results, and efforts to assess sequence quality should be a standard step in sequence-based studies. I then describe a novel approach to identify potential errors so that low quality sequences and/or suspicious bases

can be avoided. This approach is demonstrated using an alignment of approximately 500 sequences of the large subunit mitochondrial ribosomal DNA (LSU rDNA) because resources available on the Comparative RNA Web (CRW) Site (http://www.rna.ccbb.utexas.edu/, Cannone et al., 2002) make rDNA particularly amenable to this analysis. However, the general approach could be applied to any type of sequence data as long as adequate comparative data are available. This analysis indicates that sequences available in public databases vary in quality with rare instances of error occurrences greater than 10%.

Sources of Sequence Error

Errors can be introduced into published sequences in a variety of ways. 1) One source is through misincorporation of nucleotides by the polymerase during cloning, such as during PCR. The difficulty with assessing this type of error is that accurate sequencing of the resulting product will merely provide strong support for the misincorporated base. 2) Misreading of sequence data, either during manual sequencing or by base-calling software in automated sequencing, is also a possible source of error. Miscalls frequently result from insufficient electrophoretic fractionation. The most common electrophoretic anomalies are compressions, in which hairpin-like structures form near the end of a single-stranded fragment allowing the fragment to migrate faster during electrophoresis (Sanger et al., 1977). The terminal nucleotide is then interpreted as being further upstream than it actually is generating increased noise for the upstream positions. Sequences with high GC content are particularly susceptible because of their increased

likelihood to form stable hairpins (Ewing et al. 1998). Misreads also commonly result from a weak G signal following an A (Lee et al., 1992; Parker et al., 1996). 3) Mistyping during sequence and contig editing can also generate sequence errors. This problem may be particularly pronounced with manual sequencing data because errors could be introduced during the reading of autoradiographs. 4) Finally, vector sequences and other cloning artifacts can contaminate the sequence of interest (Kristensen et al., 1992; Lamperti et al., 1992; Hill et al., 2000), and laboratory or natural (e.g., parasites) contaminants can also accidentally be sequenced instead of the target individual.

Many errors are likely to be non-random. For example, secondary structure, which may cause electrophoretic anomalies, is more pronounced in rDNA resulting in increased error rates relative to other classes of DNA (Krawetz, 1989). Similarly, sequences with increased GC content are likely to have a higher error rate because of electrophoretic anomalies associated with sequence folding. Coding and non-coding sequences also are likely to vary in error rate. An initial error in coding sequence is likely to result in a nonsynonymous change or the introduction of a stop codon (the latter is especially likely with indel errors; Clark and Whittam, 1992). Thus, these initial errors are often easier to detect and correct prior to analysis resulting in a lower error rate in the final published sequence. All submissions of coding sequence to GenBank, for example, are examined for stop codons and the submitter notified if any are present.

Publicly available sequence data are from various regions of the genome and originate across a range of years, sequencing technologies, sequencing chemistries, and labs. The sources of error and the underlying error rate depend on all of these factors. The

per base cost of sequencing has declined dramatically through time. Therefore, an older published sequence may be derived from only one or two sequence reads via manual sequencing with relatively poor sequencing chemistry. In contrast, a more recent sequence (i.e., contig) may be based on multiple reads from an automated sequencer with standardized sequencing chemistry and highly accurate base-calling software. In the most extreme case, a given published sequence may be from a genome sequencing project and result from multiple independent reads from multiple clones. Nevertheless, even with automated sequencers and standardized chemistry, base calling software has an inherent error rate (Ewing et al, 1998; Ewing and Green, 1998; Johnson and Slatkin, 2007), suggesting that, regardless of how sequence data were generated, errors will occur and do exist in sequence databases.

Do these errors actually matter? Ignoring the possibility of errors may be acceptable if the level of sequence error is too low to impact the conclusions of a study, or if any impact would be within the standard deviation or confidence intervals of any parameters estimated from the sequence data. Alternatively, if results may be strongly influenced by errors, then approaches are needed for identifying and minimizing errors. Thus, the solution to this question is dependent on both the observed error rates (addressed below) and the intended use of the data (addressed under "Potential Impacts of Sequence Error").

Estimates of Sequence Error

Researchers have estimated sequence error rates for DNA polymerases as well as error rates in public database records. This latter category includes both polymerase errors and errors from other sources. Polymerase error rates have been measured by determining the number of differences between sequences from different PCR amplification reactions of the same sample. In 30-cycle PCR amplifications using *Taq* polymerase, recovered error frequencies for substitutions were 2.38 and 0.53 errors/kb (Dunning et al., 1988; Ennis et al., 1990, respectively). A slightly lower estimate of 0.3 errors/kb was suggested by Kwiatowski et al. (1991) for 40-cycle PCR amplifications with *Taq* polymerase, although this estimate was based on an extremely small sample of only four substitutions. More recently, Kobayashi et al. (1999) estimated 0.6 substitution errors/kb for 25 cycle PCR amplifications when using *Taq* polymerase and slightly lower values of 0.14 errors/kb when using a combination of *Taq* and *Pfu* polymerases (*Pfu* polymerase or combinations of *Taq* and *Pfu* give lower error rates than *Taq* alone; Kobayashi et al., 1999 and references therein). Although these estimates vary and are generally based on relatively small samples, polymerase errors clearly exist and result in 0.14–2.38 substitution errors/kb.

Corrections and revisions to sequence databases have also been used to estimate error rates of sequences in public databases. Krawetz (1989) examined revisions of GenBank sequences (which include EMBL and DDBJ sequences) and estimated 2.89 errors/kb. RNA sequences, however, were found to have a strikingly high error rate of 48.80 errors/kb, although fewer sequences were available for the RNA estimate than the

other categories examined. Clark and Whittam (1992) used a similar approach to estimate error frequencies in GenBank of 1–2 errors/kb.

Genome sequencing projects also have provided unique opportunities to assess error rates. The multifold coverage from genome sequencing provides increased confidence of low error rates (< 0.1 errors/kb; Hill et al., 2000; Wesche et al., 2004). These high-accuracy sequences can then be compared to public sequences to estimate and identify errors in the non-genome project sequences. Wesche et al. (2004) used this approach with mouse sequences and suggested 1 error/kb for coding DNA and 2.2 errors/kb for intron DNA. These values were for incorrect bases; when indels were also tabulated, the error rate increased to 4.3 errors/kb for intron DNA. The *Drosophila* genome was used in a similar study to compare genome-derived sequence with SwissProt protein sequences (Karlin et al., 2001). Of the 1,059 sequences sampled, "45% had sequence differences of more than 1%, including mismatches, insertions, and deletions." This suggests a rate of at least 4.5 errors/kb, a value that is remarkably similar to the Wesche et al. (2004) estimate. Ideally, this approach (i.e., using highly accurate sequences from genome studies to estimate error rates in public sequences) can be replicated with other genome sequences to increase our understanding of error rates in sequence databases.

Several studies have also examined sequence databases for errors due to contamination with vector sequences and other cloning artifacts. In the GenBank database, approximately 0.3% of all sequences were found to have vector contamination

(Lamperti et al., 1992; Seluja et al., 1999). Because these fragments are generally easy to avoid (see aforementioned papers), this source of error will not be considered further.

Extremely high error rates have also been reported in rare cases. Graybeal (1997) examined the phylogenetic relationships of bufonid frogs using partial small and large subunit rDNA sequences. The small subunit data were collected via manual sequencing, while the large subunit data were collected via automated sequencing. To check that both methods were generating similar results, several individuals were sequenced under both methods, and divergences up to 1.1% were recovered. However, subsequent sequencing of the large subunit for the same individuals examined by Graybeal (1997) yielded even greater differences. Harris (2001) found 2.2% sequence divergence in re-sequencing of a *Bufo calamita* and nearly 4% in *B. melanostictus* (assessed by re-sequencing the LSU rDNA of multiple individuals including one from the same locality as Graybeal's specimen). Pauly et al. (2004) found differences up to 10.2% (mean 5.2%; or 52 errors/kb) in re-sequencing the LSU rDNA of 14 Graybeal samples. Neither study found such dramatic error rates for the small subunit data that was generated with manual sequencing.

Potential Impacts of Sequence Error

The aforementioned studies suggest that overall error rates, which included polymerase errors and other sources of errors, are approximately 1–4.5 errors/kb, with rare instances of much higher rates for particular classes of sequence data (Krawetz, 1989) or for particular studies (e.g., Graybeal 1997, as described above). The critical

question is do these error rates matter for a given line of investigation? Kwiatowski et al. (1991) argued that interspecific analyses are unlikely to be significantly impacted by error rates of a few errors/kb. These authors argued that although errors may reduce support (e.g., non-parametric bootstrap support or Bayesian posterior probabilities) for weakly supported nodes or prevent the resolution of nodes for which there is little phylogenetic signal (i.e., yield polytomies), strongly supported nodes should not be impacted. However, Kwiatowski et al. (1991) did not consider impacts on likelihood model parameterization. Because A↔T, G↔C, A↔G, and C↔T errors are significantly more common than A↔C and G↔T errors (Kobayashi et al., 1999; Clark and Whittam, 1992), the error disproportionately affects certain classes in the substitution rate matrix. Estimates of rates of evolution and selection based on comparisons among synonymous and nonsynonymous substitutions and polymorphisms could also be affected (Wesche et al., 2004).

Although error rates of a few errors/kb may not be problematic in phylogenetic analyses (as suggested by Kwiatowski et al., 1989), several studies demonstrate that high error rates in particular studies have resulted in incorrect phylogenetic inferences. For example, in a phylogenetic analysis of 18S rDNA sequences, Hedges et al. (1990) recovered unexpected relationships among tetrapod lineages. Subsequently, Xia et al. (2003) sequenced additional tetrapods including multiple representatives of every major lineage sampled by Hedges et al. Surprisingly, in a combined analysis of sequences from both groups, the Hedges et al. sequences formed a clade to the exclusion of all other individuals. Xia et al. (2003) attributed this result, in large part, to poor sequence quality

and systematic errors that acted as synapomorphies for the Hedges et al. sequences. Similarly, several researchers have reported that unexpected phylogenetic relationships among toads recovered by Graybeal (1997) in her rDNA analysis are due to high sequence error (Harris, 2001, Cunningham and Cherry, 2004; Pauly et al., 2004; Frost et al., 2006). In particular, Graybeal's analysis suggested a complex biogeographic history with numerous intercontinental dispersal events because her molecular analyses failed to recover a number of now well-established geographically-delimited clades (Pauly et al., 2004, Frost et al., 2006; Pramuk, 2006). Low quality sequences from the Hedges et al. (1990) and Graybeal (1997) studies are publicly available on GenBank and have been used in multiple studies (e.g., Frost et al., 2006 used several Graybeal sequences).

Sequence errors, even at low rates, can be especially problematic for intraspecific and population genetic studies. Errors can inflate estimates of numerous parameters such as F_{ST}, θ, and the total number of haplotypes (Clark and Whittam, 1992; Kobayashi et al., 1999; Johnson and Slatkin, 2007) and be particularly problematic in identifying SNPs. Approaches in individualized medicine (reviewed by Evans and Relling, 2004), which often focus on identifying rare substitutions or polymorphisms, will also require minimal error rates.

Error Identification Using Comparative Analyses

This discussion demonstrates that researchers should pay increased attention to the possibility of sequence errors. This is especially true for sequence data from public databases because they often lack supporting information on sequence quality.

Unfortunately, few strategies and software tools are available for examining sequence quality and error rates in the absence of quality scores or chromatograms. Available programs are also only applicable to coding sequences (e.g., Brown et al., 1998; Schiex et al., 2003).

Here, I will present a method that utilizes comparative analyses to identify potential errors or anomalous bases in sequences. This general approach is feasible for any sequence type as long as an adequate number and diversity of sequences are available for comparative analyses. I will demonstrate this approach using an rDNA example for several reasons. First, Krawetz (1989) found that error rates are greater for rDNA than other types of sequence data suggesting that error assessment may be more critical for rDNA studies. Second, comparative analyses of rDNA sequences are greatly facilitated by resources available on the Comparative RNA Web (CRW) Site (Cannone et al., 2002). Third, in previous (Darst and Cannatella, 2004; Pauly et al., 2004) and ongoing phylogenetic research, the same individuals and gene region were sequenced as in an earlier study (Graybeal, 1997); extremely high sequence divergences were recovered between sequence pairs from the same individuals indicating high error rates in the earlier sequences and providing a unique dataset to examine errors and methods of assessing them.

The basic premise of this approach is that sequence anomalies and/or low quality sequences can be detected by comparing the nucleotide of interest to homologous nucleotides across a larger dataset (Fig. 4.1). For example, many rDNA nucleotide positions are highly conserved across the Tree of Life. Sequences with substitutions at

these conserved sites can be flagged as being anomalies (or suspected errors). In other words, sequence conservation information is used to assess the potential for observing a substitution in the sequence of interest. Although these flagged or anomalous bases may be true mutations (e.g., apomorphies), an unusually large number of anomalies in one sequence suggests sequencing error. In this case, “an unusually large number of anomalies” can be interpreted relative to the other sequences available for the clade of interest, such as for sequences from other sources (i.e., different labs or different sequencing technologies). I will refer to potential errors as "anomalies," with the idea that errors can only be truly confirmed through re-sequencing. Sequences with unusually high numbers of atypical nucleotides at highly conserved sites can then be flagged as potentially having a high error rate and excluded from analyses.

rDNA sequences are ideal candidates for such analyses for several reasons. First, comparative studies of rDNA have identified patterns of sequence conservation and variation at the level of primary and secondary structure, and this information is publicly available on the CRW Site. In particular, sequence conservation information, which is critical to this approach, has already been tabulated from large secondary structure-based alignments that include representatives from throughout the Tree of Life. Further, seed alignments and secondary structure models for diverse organisms allow researchers to rapidly align their dataset to the *Escherichia coli* sequence that serves as a reference to this sequence conservation information. Second, many nucleotide positions in rDNA sequences are highly conserved across all life, providing a large sample for identifying anomalies in test sequences. Finally, rDNA sequences can be aligned using secondary

structure information (Gutell et al., 1985), which provides increased confidence in the alignment and homology assessment (Kjer, 1995; Hickson et al., 2000; Kjer et al., 2007); this feature is especially important given the need to align across diverse lineages and from the test group to the *E. coli* reference sequence.

ASSESSING SEQUENCE ERRORS: AN EXAMPLE FROM TOADS

4.2 METHODS

This method for assessing sequence anomalies is demonstrated through an analysis of the mitochondrial large subunit rDNA from numerous bufonids, a speciose and cosmopolitan anuran family. The sedimentation coefficient "16S" correctly refers to both small (prokaryotes, eukaryotic chloroplast) and large (animal mitochondria) subunit rDNAs; to avoid confusion, throughout this manuscript I will use the terms "large subunit" (or LSU) and "small subunit" (or SSU) rDNA to refer to the two rDNAs, regardless of their taxonomic position or organellar identity.

A comparative dataset was generated by selecting all bufonid mitochondrial LSU rDNA sequences from large phylogenetic studies of anurans or studies that specifically focused on bufonids. These included Graybeal (1997), Liu et al. (2000), Mulcahy and Mendelson (2000), Gluesenkamp (2001), Cunningham and Cherry (2004), Darst and Cannatella (2004), Pauly et al. (2004), Frost et al. (2006), Kutrup et al. (2006), Pramuk (2006), and Matsui et al. (2007). Additional mitochondrial LSU rDNA bufonid sequences

were selected from GenBank to increase the diversity of taxa, studies, and research groups represented. Finally, four individuals recently sequenced as part of ongoing research on bufonids (by Pauly and D. C. Cannatella, in progress) were also included because these same individuals were previously sequenced by Graybeal (1997); their inclusion increased the number of individuals for which direct counts of the number of errors in the earlier sequences are available. Methods for extraction, amplification, and sequencing follow Pauly et al. (2004).

The alignment of Pauly et al. (2004; n = 82 individuals), which was based on maximizing sequence and secondary structure conservation, was used as a seed alignment for generating the full alignment. Sequences were added to this preliminary alignment by aligning them to close relatives using the pair wise alignment tool in MacClade (Maddison and Maddison, 2000). The dataset was then aligned by eye both internally and to reference sequences available on the CRW Site (*E. coli, Xenopus laevis, Rana catesbeiana,* and the 3 Phylogenetic Domain/2 Organelle (3P20) conservation sequence; see below for a description of the conservation sequence) in the alignment editor AE2 (developed by T. Macke, Scripps Research Institute, San Diego, CA; Larsen et al., 1993), which runs on Sun Microsystems workstations with the Solaris operating system. The non-bufonid reference sequences were used to propagate conserved secondary structure motifs in the bufonids and to aid in alignment to the conservation sequence.

The 3P20 conservation sequence is of particular importance to this analysis and is described here in greater detail. The conservation sequence summarizes nucleotide conservation from a large alignment (930 sequences) with representatives from

throughout the Tree of Life. This sequence is termed the 3 Domain/2 Organelle conservation sequence because the alignment from which it is derived includes representatives of Archaea, Bacteria, and Eukarya (the three phylogenetic domains) as well as the mitochondrial and chloroplast rDNAs (the two eukaryotic organelles). Positions that are the same nucleotide (A, C, G, or U) in 98% or more of the sequences in the 930-sequence alignment appear in capital letters while individuals with 90–98% conservation appear in lower case letters (Fig. 4.1). Positions with <90% conservation are treated as ambiguous (N). The position number of each nucleotide in the *E. coli* reference sequence and the 3P20 conservation sequence can be used to look up more detailed sequence conservation information on the CRW Site (Fig. 4.2).

The finished bufonid AE2 alignment was then exported to MacClade. Most of the sequences in the bufonid alignment did not provide data for the entire LSU rDNA sequence. Not surprisingly, the 5' and 3' ends of some sequences had mismatches when compared to longer sequences. Because the sequence ends are often excluded from analyses because of low sequence quality, the ends of any sequences with unexpected mismatches were also deleted from the main alignment so as not to inflate the number of observed anomalies with positions that were likely excluded from the original analyses. This problem was especially pronounced for some of the Graybeal sequences which had so many errors in their 5' and 3' ends that they were unalignable even to newer sequences of the same individual; thus, an even greater number of nucleotides were excluded from the ends of these sequence.

Sequences were then examined for anomalies. The 3P20 conservation sequence was used to make two pared-down datasets containing only positions with high sequence conservation across the Tree of Life. The first dataset included only those positions with ≥90% sequence conservation in the 3P20 alignment. The second was a further reduction that only included positions with ≥98% conservation. The ≥90% and ≥98% datasets were then examined for anomalies by generating distance matrices in PAUP* (Swofford, 2003) to determine the number of nucleotides in each sequence that differ from the typical nucleotide for that position as given in the conservation sequence. The result is a list of the number of non-standard nucleotides found in all positions with 90–100% sequence conservation and 98–100% sequence conservation for every sequence in the alignment. The occurrence and location of these nucleotides in the alignments can be easily visualized by using the "color-cells-matching-first-taxon" option in MacClade with the 3P20 conservation sequence as the first taxon.

The large bufonid dataset was used to explore the occurrence of bases inconsistent with the 3P20 conservation sequence. A more explicit investigation was conducted using a unique dataset derived from re-sequencing numerous individuals originally sequenced by Graybeal (1997). Re-sequencing allows a determination of the error rates in the Graybeal sequences and provides sequences with known errors that can then be analyzed using the comparative approach developed here. The number of errors in each of 33 publicly available LSU rDNA sequences from Graybeal (1997) was directly counted using distance matrices in PAUP* by comparing the older sequences to recent sequences of the same individuals (and same tissue samples) or individuals from the same or nearby

populations. Although a number of the Graybeal sequences had insertions and deletions in comparison to their newer counterpart, these indels were not counted in calculating sequence divergence (i.e., error rate). Additionally, as in the larger alignment, the ends of many of Graybeal's sequences were excluded. As a result, the reported error rate is actually a conservative estimate.

The 33 re-sequenced individuals are from Pauly et al. (2004; 21 individuals), Darst and Cannatella (2004; 9 individuals), or recently sequenced specimens (3 individuals). Of these 33 new sequences, 25 are of the same individuals examined by Graybeal (1997), 3 are from the same locality as their Graybeal pair, and 5 are conspecifics from localities near to the locality of their Graybeal pair (Table 4.1). Therefore, for the eight non-identical samples, a very small amount of sequence divergence is possible due to intraspecific variation, although such variation would be expected in variable regions (e.g., loop regions) and not in the highly conserved positions from the 3P20 conservation sequence.

An identical analysis was performed for the small subunit mitochondrial rDNA (SSU rDNA) sequences. Graybeal's SSU rDNA sequences were generated with manual sequencing and have been reported to have lower error rates (Harris, 2001; Pauly et al., 2004). Three of the specimens from the LSU rDNA analysis lacked SSU rDNA data, so only 30 sequence pairs were available for the SSU analysis.

Interpretation of anomalies is demonstrated with reference to Figure 1. This alignment shows positions 1906 to 1934 of the *E. coli* reference sequence and eight hypothetical sequences from six species and two studies (Fig. 4.1a); this region includes a

stem-loop feature and numerous highly conserved nucleotides (Fig. 4.1b). At position 1911, two anomalies are recovered in the two species from Study 2 (U→G), but their respective conspecifics from Study 1 lack these atypical nucleotides. Further, on the opposite side of the loop (marked with parentheses in Fig. 4.1a), the paired nucleotide (position 1918) does not show covariation to maintain the pairing in the stem. Because these anomalies occur at positions with ≥ 98% sequence conservation, only occur in sequences from one study, and lack covariation in their structural pair, they are very likely to be errors.

Anomalies are also recovered at position 1910 of the alignment (G→A; Fig. 4.1a). Although this anomaly is at a well-conserved position (90–98% sequence conservation), multiple sequences from both studies recover an A at this position. Further, this site is part of a stem region, and covariation at position 1919 (i. e. C→U) maintains the Watson-Crick pairing in the stem. Therefore, this anomaly is likely to be a real synapomorphy for a clade that includes these species.

Another likely error is shown at position 1934 (G→A), where only one individual has a substitution at a position with ≥98% sequence conservation in the 3P20 alignment when numerous closely related species, including a conspecific, have the expected nucleotide. The substitution at positions 1935, in which species 2–6 from both studies record a substitution (C→G), is much more likely to be real.

4.3 Results

The LSU rDNA alignment included 489 bufonid sequences that varied in the total number of nucleotides from 336 to 1452. This region includes 303 characters with ≥90% sequence conservation, of which 97 characters have ≥98% sequence conservation in the 3P20 alignment.

Numerous anomalies were found in Graybeal's sequences (Fig. 4.2a, 4.3), including insertions (e.g., *B. terrestris* and *B. microscaphus* between positions 2500 and 2501; *B. cognatus* between positions 2509 and 2510), deletions (e.g., positions 2501 to 2503 in *B. bocourti*), and substitutions at highly conserved positions. For example, numerous Graybeal sequences report an A at position 2501 (Fig. 4.2a), but as depicted in the nucleotide frequency table in Fig. 4.2b (modified from the CRW Site), 99.64% of the 829 sequences in the 3P20 alignment with sequence data for this site, including all 306 mitochondrial sequences, have a C at this position, indicating that the recovered A's likely represent systematic error. Re-sequencing confirmed that these anomalous A's and all other anomalies depicted in Fig. 4.2a were true errors.

For the 33 Graybeal LSU rDNA sequences, the old (Graybeal) and new sequences overlapped for up to 499 nucleotides. The mean pair wise difference was 4.96% (range 0–14.27%), meaning that the error rate was up to 143 errors/kb (Table 4.1). Pair wise differences for the SSU rDNA were much lower. The area of overlap was approximately 950 bases for most pairs, although a few were shorter because the Graybeal sequence was incomplete on one end (usually the 3' end). The mean sequence divergence was 0.3%

(range 0–0.93%; Table 4.1). Some differences in SSU sequence pairs may be attributable to polymerase errors in the newer sequences or true differences in the seven non-identical pairs. Nevertheless, even if all differences between SSU rDNA sequence pairs are attributed to errors in the older sequences, the SSU sequences would have a mean error rate (3 errors/kb) within the typical range of error rates (1–4.5 errors/kb).

Comparative analyses using the conservation information readily identified the high error rate in the Graybeal LSU rDNA sequences. The Graybeal sequences varied in the number of nucleotides available for analysis because they 1) varied in length, 2) had varying numbers of bases identified as ambiguous, and 3) had differing numbers of nucleotides excluded from the ends of each sequence because they were unalignable to their new sequence pair. Nevertheless, the Graybeal sequences included 52–63 positions with ≥98% sequence conservation in the 3P20 alignment (Table 4.1); up to 14 of these positions were found to be inconsistent with the conservation sequence (i.e., anomalous). Importantly, none of the recovered anomalies at the 98% conservation threshold were validated by the new sequences, demonstrating that they are all true errors. Also, some errors were systematic in that the same erroneous nucleotide occurred in different sequences at the same position (Fig. 4.2a), which might lend support to artificial groupings.

At the less restrictive threshold of 90% sequence conservation, the Graybeal sequences included 122 to 148 positions (Table 4.1). Substitution anomalies were found in up to 37 of these positions (Table 4.1). Re-sequencing confirmed that all of these anomalies are true errors except for one position in five individuals. Position 2518 is an A

in 93.2% of sequences in the 3P20 alignment (specific percentages for each nucleotide of each position in the 3P20 alignment are available on the CRW Site). However, only 85.9% of the mitochondrial sequences in the 3P20 alignment are an A. In the bufonids, many species have a C or U at this position. This situation demonstrates one way of confirming errors. Even if re-sequencing is not possible, the occurrence of atypical nucleotides in the target sequence's close relatives and/or the lower conservation in the mitochondrial sequences of the 3P20 alignment can indicate the atypical nucleotide is not an error (see also Fig. 4.1).

The Graybeal sequences have the highest occurrence of anomalies across the entire bufonid dataset (Fig. 4.3). In addition to the 33 Graybeal sequences examined through re-sequencing, there are 14 additional LSU rDNA bufonid sequences from Graybeal (1997). These had 0 to 21 anomalies in positions with ≥90% sequence conservation. Other sequences in the bufonid alignment also had unexpected numbers of anomalous nucleotides, including up to 8 anomalies in positions with ≥90% sequence conservation (Fig. 4.3). I will discuss some of these anomalies in greater detail to demonstrate strategies for determining whether anomalies are likely errors or true mutations.

As described previously, comparisons to close relatives can help determine whether or not anomalies are likely to be errors. The genus *Ansonia* is represented by nine sequences from five different studies (not including the single *A. muelleri* from Graybeal, 1997). These nine sequences have 2–8 anomalies in positions with ≥90% sequence conservation. However, many of these anomalies are shared among multiple

sequences from different studies, suggesting that they are not errors but true synapomorphies for the genus or a clade within the genus. If these shared anomalies are disregarded because they are unlikely to be errors, then only four of the sequences still have anomalies. Three sequences have 1 anomaly each and one sequence has 3 anomalies. Further assessing whether these remaining anomalies are true errors is complicated by the relatively high percent sequence divergence among *Ansonia* species, which increases the plausibility of rare apomorphies in highly conserved positions.

As was observed for the Graybeal SSU and LSU datasets, error rates are likely to vary with methods of sequence data collection. This suggests that error rates also are likely to vary with studies, researchers, and/or laboratories. Several studies containing sequences with unusually high numbers of anomalies were uncovered. Both bufonid sequences from Singh and Prakash (2006) and a number of sequences from Gluesenkamp (2001) had high numbers of anomalies relative to sequences from conspecifics or close congeners from other sources (Table 4.2).

A similar example of a sequence dataset with unusually high numbers of anomalies involves a phylogeography study. Kutrup et al., (2006) examined intraspecific variation in *Bufo bufo* from Turkey and recovered 19 haplotypes. The *B. bufo* sequences were approximately 965 bases long and included 210 positions with ≥90% sequence conservation. Examination of the highly conserved positions found a surprising number of anomalies in these sequence haplotypes but not in two other *B. bufo* from other studies or 11 other representatives of the *B. bufo* species group. Ten individuals had 1–2 unique substitutions at positions with ≥90% sequence conservation (for a total of 13 anomalies)

and 5 of these were at positions with ≥98% sequence conservation. If these anomalies are errors, then the number of inferred haplotypes is greatly overestimated. By conducting this comparative analysis, researchers hoping to include *B. bufo* in their own study could now avoid these potentially problematic sequences in favor of other publicly available sequences that lack such anomalies.

4.4 DISCUSSION

Given the rarity of formal examinations of sequence quality, the unstated assumption of many sequence-based studies seems to be that sequence error is small and has an insignificant effect on results. However, even simple consideration of a conservative error rate demonstrates this assumption is often false. Consider an extremely low error rate of 0.1 errors/kb. Such a low rate would merely introduce the occasional apomorphy in interspecific studies. However, the occasional error could be much more damaging in datasets with little variation (i.e., signal) such as in many intraspecific studies. In a phylogeographic study, a moderately sized dataset of 100 individuals with 1kb of sequence data per individual would have 10 errors. These 10 errors likely result in 10 unique haplotypes, which would dramatically inflate many commonly measured parameters (e.g., the number of private alleles, the number of haplotypes, or the population genetic parameter θ). Clearly, even low error rates can influence the results of some studies.

Unfortunately, error rates as low as 0.1 errors/kb are unlikely to be achieved in currently available sequences except with the multifold coverage of genome sequencing projects (Hill et al., 2000). As reviewed previously, error rates are likely to be 1–4.5 errors/kb. Further, available estimates are averages, and while these are informative, much more worrisome are the upper extremes. The highest rates uncovered in this study exceeded 100 errors/kb (Table 4.1). Presumably such a high rate is extremely rare and likely reflects the method of sequence generation. These sequences were generated on an early automated sequencer, and the frequent errors are presumably associated with the early and unfamiliar technology. The manually generated sequences from the same study had a much lower error rate that was on par with standard estimates of error (Table 4.1). Although error rates as high as those observed for the Graybeal LSU rDNA sequences may be rare, the analysis of all bufonids based on highly conserved positions found likely errors in sequences from a variety of studies, including some that are relatively recent. These examples demonstrate that at least some publicly available sequences from various studies, laboratories, and/or sequencing technologies have error rates well above standard estimates.

This observation is worrisome as thousands of studies each year rely on sequences downloaded from public databases. These sequences generally lack supporting information that indicates data quality. Therefore, without assessments of sequence quality, sequences with errors can go unnoticed and be incorporated into additional studies. This exact scenario has occurred for Graybeal's error prone sequences and the phylogenies derived from them. For example, several studies have included multiple high

error rate Graybeal sequences in their analyses (e.g., *B. coniferus*, *B. alvarius,* and *B. fastidiosus* in Mulcahy and Mendelson [2000], and *Bufo mazatlanensis* and *Ansonia muelleri* in Frost et al. [2006]); other studies have used the phylogenies from Graybeal's (1997) analysis to assess character state evolution (e.g., da Silva and Mendelson, 1999; Summers et al., 2006; 2007). Importantly, these authors did not do anything differently than other researchers; they merely were unlucky in that the sequences or phylogenies they incorporated happened to have high sequence error.

The inclusion of these sequences has varied impacts on the results of these studies. Mulcahy and Mendelson (2000) used the sequences as several of their outgroup species; although the relationships among their outgroup species were different than the relationships found in more recent studies lacking these error-prone sequences (Pauly et al., 2004; Pramuk, 2006), the position of the root for their ingroup is consistent across studies. Therefore, for this study, the inclusion of the high error rate sequences appears to have minimal impact. In contrast, Graybeal's (1997) phylogeny suggests a rather different reconstruction for the number and sequence of gains and losses for the characters of interest in da Silva and Mendelson (1999) than would be suggested by other phylogenies not using these sequences (Pauly et al., 2004; Pramuk 2006). Although the specific impact that these sequences have on these studies is beyond the scope of this work, these examples demonstrate that error-prone sequences exist in public databases and are uncritically incorporated into studies.

To avoid error-prone sequences, screens for sequence error should be a regular step in sequence-based analyses, but easily implementable programs and strategies are

necessary. Several programs exist for examining sequence quality in coding regions (e.g., Brown et al., 1998; Schiex et al., 2003). Here, I presented a straightforward approach for detecting low quality sequences in RNA datasets, and the general methodology could also be applied to other coding and non-coding regions.

This method identifies sequences with a large number of unexpected substitutions in highly conserved positions. This approach does not identify all potential errors in a sequence. Errors in difficult to align regions will not be identified because the test dataset is restricted to areas of higher sequence conservation. Therefore, an investigator will have to estimate a whole sequence error rate based on the number of anomalies in the nucleotides that can be assessed. Each investigator will then have to decide how to proceed based on the goals of their study. In some cases, the presence of an anomaly may demand additional sequencing and/or the exclusion of the sequence in question. Additional sequencing is particularly relevant when there are few closely related sequences available and the question of whether observed anomalies are errors or genuine mutations remains. Surprising results should not merely be discarded, as the presence of a large number of anomalies may also indicate a high rate of evolution or other processes worthy of further study. In cases where closely related sequences are available and there is increased confidence in treating anomalies as errors, then these other publicly available sequences lacking suspected anomalies may be appropriate substitutes. In some phylogenetic analyses, only the most egregious cases may be worth excluding if the potential error rate is too low to overshadow data signal.

In the screen demonstrated here, positions with ≥90% sequence conservation across all life (i.e., the 3P20 alignment) were used as a test sample for finding likely errors. However, positions with lower sequence conservation may still be informative if they are well conserved in more restrictive phylogenetic partitions. Therefore, screens can also be conducted across narrower phylogenetic or organellar units such as within all Eukarya or all mitochondrial LSU rDNAs. In the case of commonly sequenced human genes (rDNA or others), screens could even be across all human sequences available. For rDNA sequence, secondary structure information can also be incorporated and similar analyses done using base pairing information, which is also available on the CRW Site. Unexpected pairs, pairs that are unlikely to form a stem, lack of covariation, and indels in stem regions all indicate potential errors (Fig. 4.1).

The key to this comparative approach is sequence conservation information for the region of interest. For rDNA, such information is available on the CRW Site in the form of conservation sequences and affiliated nucleotide frequency tables that summarize large datasets. In particular, the 3P20 conservation sequence allows rapidly assessing variation in highly conserved positions. As part of this research, the 3P20 conservation sequences of the large (summed across 930 sequences) and small subunit rDNAs (summed across 7355 sequences) are now available on the CRW Site. Therefore, researchers wanting to assess anomalies in their large and small subunit datasets merely have to add the 3P20 conservation sequence to their alignment and then assess anomalies in the highly conserved positions. Adding this sequence is no more complicated than downloading and adding a sequence from another sequence database. In cases where

alignment of the test dataset to the 3P2O conservation sequence is complicated by low sequence similarity, seed alignments can also be downloaded to expedite the process. Conservation sequences for other genes and taxonomic divisions are also available in the numerous secondary structure models on the site.

For other regions of the genome, per site nucleotide frequencies and the conservation sequences that summarize this information will have to be generated to screen for anomalies. In developing such datasets, researchers should aim to maximize the number and taxonomic diversity of sequences included to ensure that the conservation information is representative of the true variation in each position.

Another consideration is the evolutionary history of the site being examined. Sequence conservation, the main statistic employed here, indicates the number of sequences with an atypical nucleotide, but it does not indicate the number of times that an atypical nucleotide has evolved. Therefore, a position that is 100% conserved within each phylogenetic domain may have a low conservation score if in each domain a different nucleotide is present. In developing new datasets or expanding on the current rDNA framework, gathering information on the consistency index would also be informative.

A final avenue of research that would be beneficial to understanding error rates is to consider how these rates fluctuate as technology changes. Unfortunately, there are few recent studies of error rates. Most studies of sequence error are old enough that they deal with data from outdated sequencing technologies. However, the number of bases in GenBank doubles approximately every 18 months (GenBank release 166 [June 15, 2008], Distribution Release Notes; ftp://ftp.ncbi.nih.gov/genbank/gbrel.txt). As a result, most

sequence data are from more recent sequencing technologies. The use of standardized amplification and sequencing chemistries, increased accuracy of base calling software, and a larger number of reads per finished sequence should decrease error rates. New studies estimating error rates of recently generated sequences would be very useful for interpreting the impact of sequence errors.

TABLE 4.1. Assessment of errors in Graybeal's (1997) large (LSU) and small subunit (SSU) mitochondrial RNA sequences. The percent sequence divergence is between Graybeal's (1997) sequences and a more recent sequence of the same individual or geographically proximate conspecific. This divergence is interpreted as the sequence error rate. The total number of positions in each large subunit sequence with ≥98% and ≥90% sequence conservation in the 3 Domain/2 Organelle alignment is also given along with the number of positions for which Graybeal's sequences suggest a non-standard nucleotide.

Species	Genbank No. Graybeal Sequence (LSU only)	Genbank No. New Sequence (SSU and LSU)	% Seq. Div. LSU (automated sequencing)	% Seq. Div. SSU (manual sequencing)	Positions with ≥ 98% Conservation			Positions with ≥ 90% Conservation		
					Total No.	No. Anoms.	% Anom.	Total No.	No. Anoms.	% Anom.
Bufo terrestris	U52767	AY680220	14.27	0.11	52	12	23.08	124	21	16.94
Bufo mauritanicus	U52758	AY680265	11.55	0.00	56	12	21.43	140	33	23.57
Bufo microscaphus[2]	U52764	AY325989	11.47	0.00	60	14	23.33	144	37	25.69
Bufo bocourti	U52757	AY680245	10.98	0.00	59	13	22.03	137	24	17.52
Bufo canorus[1]	U52756	AY680239	10.31	0.65	57	6	10.53	135	16	11.85
Bufo debilis	U52778	AY680233	8.66	0.11	54	7	12.96	122	12	9.84
Bufo exsul	U52753	AY680243	7.65	0.22	54	8	14.82	127	14	11.02
Bufo fastidiosus	U52788	AY680248	7.34	0.44	55	7	12.73	132	12	9.09
Bufo boreas	U52752	AY680244	7.19	0.11	58	5	8.62	143	10	6.99
Bufo fowleri	U52771	AY680224	6.85	0.59	57	9	15.79	140	18	12.86
Bufo taitanus	U52765	EU938402	6.24	0.00	58	5	8.62	142	13	9.16
Bufo granulosus	U52795	AY680261	6.07	0.38	58	10	17.24	140	18	12.86
Bufo retiformis	U52781	AY325982	5.57	0.37	58	5	8.62	143	9	6.29
Bufo punctatus	U52785	AY680237	5.40	0.34	58	3	5.17	143	10	6.99
Bufo speciosus	U52777	AY680228	5.16	0.22	58	8	13.79	142	14	9.86
Bufo haematiticus[2]	U52791	AY680270	4.47	0.73	57	4	7.02	141	11	7.80
Bufo cognatus	U52774	AY680230	4.43	0.00	57	5	8.77	138	11	7.97
Bufo asper	U52772	AY680266	4.34	0.54	55	5	9.09	128	14	10.94
Bufo gariepensis	U52768	EU938401	3.57	0.11	62	7	11.29	147	17	11.57
Bufo mazatlanensis	U52755	AY680254	3.40	0.00	58	4	6.90	143	11	7.69
Bufo melanochlorus	U52793	AY680255	3.40	0.00	62	4	6.45	147	13	8.84
Bufo nebulifer[2]	U52799	AY325985	3.30	0.11	58	6	10.35	142	11	7.75
Bufo quercicus	U52769	AY680235	3.28	0.55	55	2	3.64	128	4	3.13
Bufo americanus	U52760	AY680211	2.33	0.44	63	5	7.94	148	6	4.05
Bufo coniferus	U52800	AY680247	2.06	N/A	63	6	9.52	148	14	9.46
Bufo calamita[1]	U52759	EU938400	1.70	0.68	57	0	0.00	138	2	1.45
Ansonia muelleri	U52784	AY325992	0.84	0.35	57	1	1.75	139	5	3.60
Atelopus varius[2]	U52779	AY325996	0.83	0.93	58	0	0.00	142	2	1.41
Bufo tacanensis	U52754	AY680258	0.42	N/A	57	1	1.75	138	1	0.73

Osornophryne guacomayo[1]	U52783	AY326036	0.41	0.48	57	1	1.75	139	3	2.16
Bufo kisoloensis	U52773	AY325995	0.21	0.47	57	0	0.00	139	1	0.72
Bufo steindachneri[2]	U52763	AY325981	0.0	N/A	57	0	0.00	139	1	0.72
Melanophryniscus stelzneri	U52782	AY325999	0.0	0.11	57	0	0.00	140	1	0.71

[1]More recent sequence is from a conspecific from the same population as Graybeal's sample.
[2]More recent sequence is from a conspecific from a locality close to Graybeal's sample.

TABLE 4.2. Sequences with high numbers of anomalies from two studies. Only anomalies that were not found in conspecifics or close congeners are reported. Anomalies are reported as the total number at positions with 90–100% sequence conservation in the 3 Domain/2Organelle alignment / the number of these that were at positions with ≥98% sequence conservation. The numbers of comparative sequences from conspecifics and close congeners are also provided.

Species	Genbank No.	No. postions with ≥ 90 conservation	No. anomalies at Positions with ≥ 90/98% conservation	No. sequences of conspecifics and close congeners	Other suggestions of low sequence quality
Bufo melanostictus	EU367009[1]	150	3/3	13 and 1	2 deletions at positions with ≥90 conservation
Bufo stomaticus	EU367010[1]	148	6/3	0 and 0	3 deletions at positions with ≥90 conservation
Bufo biporcatus	AF375512[2]	149	2/2	1 and 4	3 deletions and 4 ambiguous bases at positions with ≥90 conservation
Nectophrynoides tornieri	AF375520[2]	67	2/2	0 and 3	
Rhamphophryne macrorhina	AF375532[2]	66	2/2	0 and 3	
Dendrophryniscus brevipollicatus	AF375515[2]	196	3/0	0 and 6	2 ambiguous bases at positions with ≥90 conservation
Pedostibes hosei	AF375529[2]	183	2/1	4 and 2	3 deletions at positions with ≥90 conservation
Pelophryne brevipes	AF375530[2]	95	2/0	1 and 1	2 deletions at positions with ≥90 conservation
Oreophrynella quelchii	AF375521[2]	67	2/2	1 and 0	

[1]From Singh and Prakash, 2006.
[2]From Gluesenkamp, 2001.

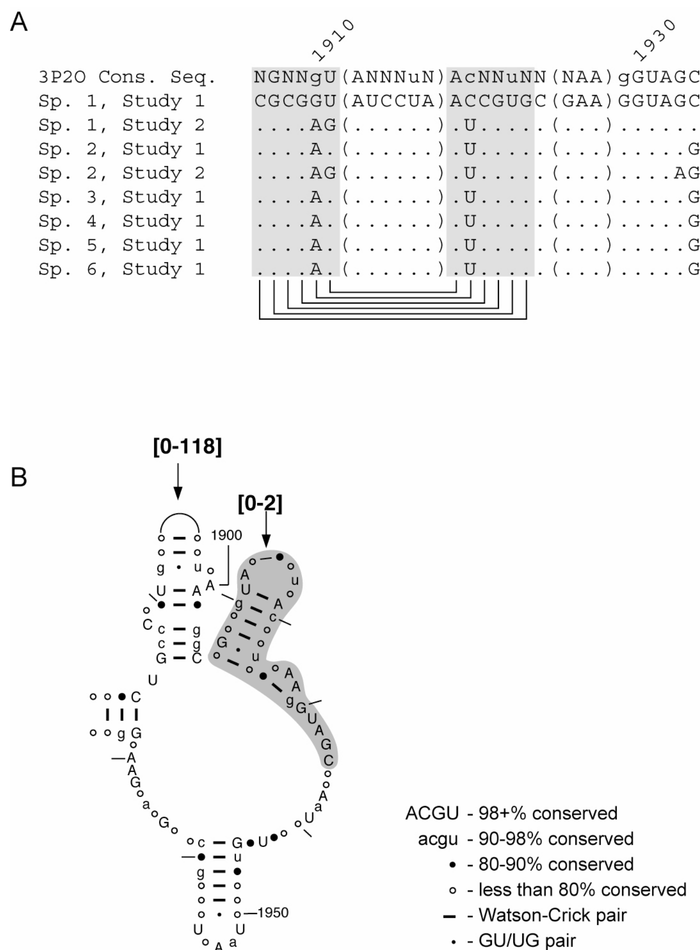
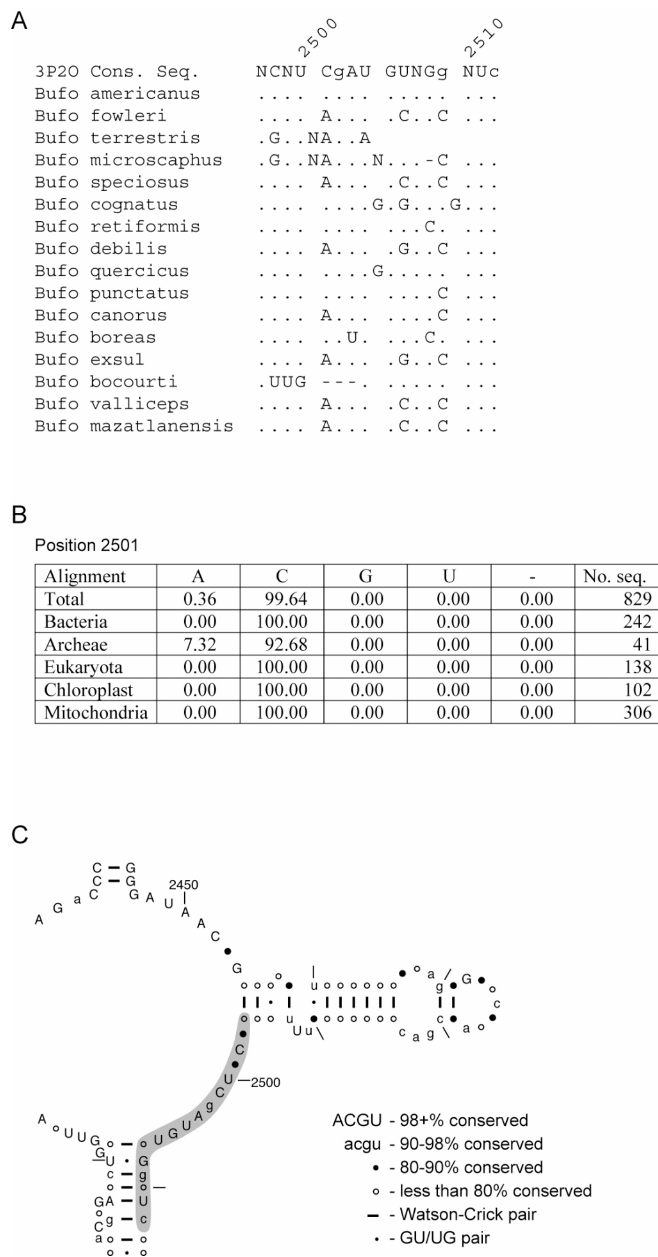


FIGURE 4.1. Examples of assessing anomalies through comparative analyses of highly conserved positions. A) Alignment of positions 1906–1934 for eight hypothetical sequences from two studies. Dots in the aligned taxa indicate agreement with the Species (Sp.) 1, Study 1 sequence. Parentheses note a loop region with the six nucleotides in gray boxes 5' and 3' of the loop forming a stem. In the 3 Phylogenetic Domain/2 Organelle (3P20) conservation sequence (top row of alignment), capital and lower case letters represent nucleotides with ≥ 98% and 90–98% sequence conservation, respectively, in an alignment of 930 sequences with representatives throughout the Tree of Life. Positions with <90% sequence conservation are given as N's. and correspond to the open or closed circles in the secondary structure diagram. B) Actual secondary structure conservation diagram excerpt with the shaded region showing the nucleotides in the alignment (A). Sequence conservation and pairing notation for the structure model is given in the key (adapted from the Three Phylogenetic Domains/Two Organelles Conservation Diagram available at the CRW Site; http://www.rna.ccbb.utexas.edu/ SAE/2B/ConsStruc/).



Alignment	A	C	G	U	-	No. seq.
Total	0.36	99.64	0.00	0.00	0.00	829
Bacteria	0.00	100.00	0.00	0.00	0.00	242
Archeae	7.32	92.68	0.00	0.00	0.00	41
Eukaryota	0.00	100.00	0.00	0.00	0.00	138
Chloroplast	0.00	100.00	0.00	0.00	0.00	102
Mitochondria	0.00	100.00	0.00	0.00	0.00	306

FIGURE 4.2. Small alignment of Graybeal (1997) sequences that is representative of the numerous anomalies recovered in these sequences through comparative analysis. A) Alignment (partial) of positions 2497–2512 for 16 Graybeal sequences and the 3 Phylogenetic Domain/2 Organelle (3P2O) conservation sequence (see Fig. 4.1 legend for notation information). Dots in the aligned sequences indicate agreement with the 3P2O conservation sequence, and dashes indicate deletions. All anomalies (substitutions, insertions, and deletions) depicted in the Graybeal sequences were confirmed to be errors through re-sequencing. B) The nucleotide frequency table for position 2501 (modified from the CRW Site); of the 930 sequences in the 3 Domain/2 Organelle alignment, 829 had data covering this position. C) Actual secondary structure conservation diagram excerpt with the shaded region corresponding to the alignment (A). Sequence conservation and pairing notation for the structure model is given in the key provided.

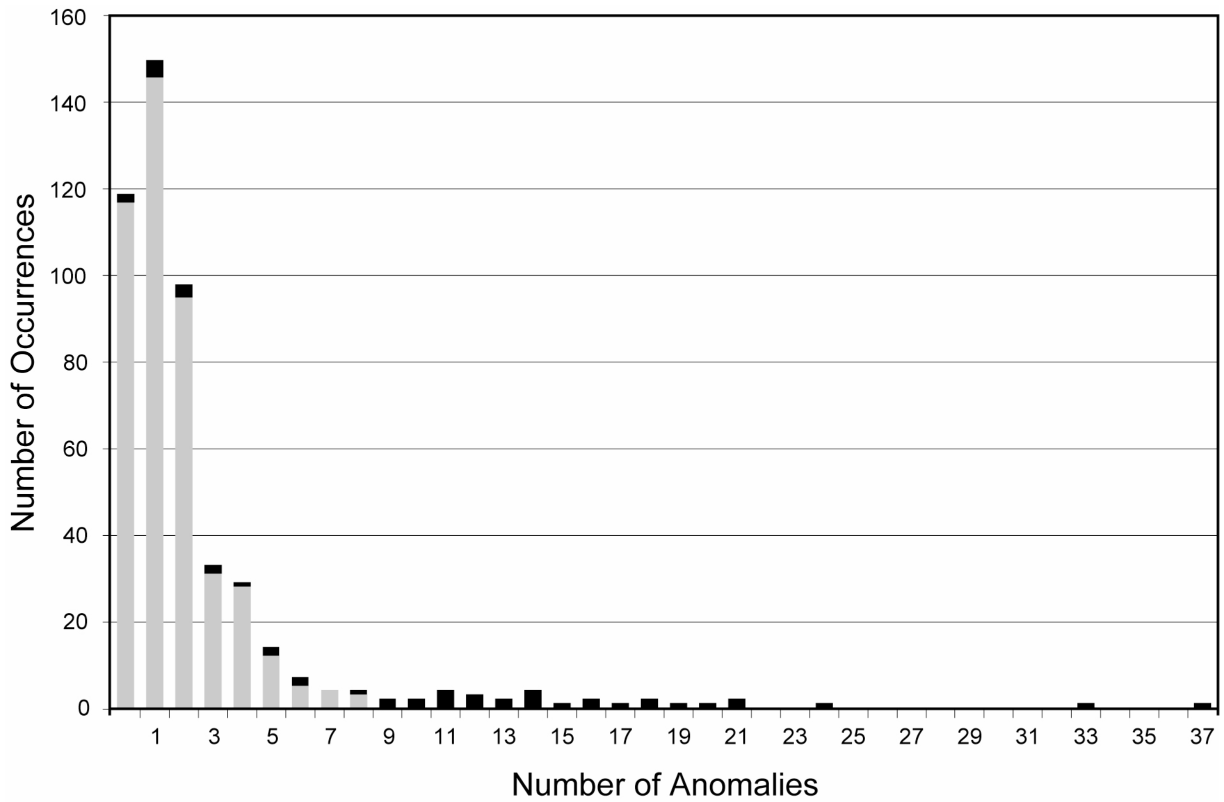


FIGURE 4.3. Histogram of the number of anomalous nucleotides found in the 489 bufonid sequences examined (indel events were not counted as anomalies in this analysis). The black portions of each bar represent sequences from Graybeal (1997; n = 47).

APPENDIX A. 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 B; 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	Louisiana: 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	AY680225
Bufo fowleri (7)	americanus	TNHC 61997	New York: Suffolk: Brookhaven Township: Gordon Heights: 0.8 km NE of intersection of Granny Rd. at 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
Bufo speciosus (35; TX-1)	cognatus	MVZ143046	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	AY680234

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)		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: ~14km 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
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

APPENDIX B. 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 A. For all parametric bootstraps, taxa 14 and 29 were excluded because they are identical to other taxa in the analysis (see Methods). Some species in the *B. americanus* group were represented by several closely related sequences 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);

APPENDIX C. Localities, identification numbers, and GenBank numbers for specimens sampled in the mitochondrial (Cyt b) and nuclear (Rhodopsin) DNA analyses. In the Rhodopsin column, numbers in parentheses indicate the number of *B. boreas* specimens examined for that locality; GenBank numbers are only provided for each unique haplotype per population. Only one individual per population was examined in the mitochondrial analysis.

Popn.	Identification No.	Locality	Genbank No.	
			Cyt *b*	Rhodopsin
Bufo boreas				
1	GBP 181	Alaska: Dyea: Taiya River Flats	EU938403	FJ004238
2	UAM 271	Alaska: vicinity of Skagway	EU938404	
3	GBP 198	Alaska: N of Juneau, near Echo Cove	EU938405	
4	GBP 239	Alaska: Taku River, ca. 4.7 km WSW of US-Canada border	EU938406	
5	UAM 23	Alaska: Yakobi Island	EU938407	
6	UAM 259	Alaska: Admiralty Island	EU938408	
7	GBP 204	Alaska: Admiralty Island: Freshwater Lake	EU938409	
8	UAM 14	Alaska: Baranof Island	EU938410	
9	GBP 260	Alaska: Wrangell Island: 16 km S of Wrangell on Zimovia Hwy	EU938411	
10	MVZ 225512	Alaska: Annette Island: Metlakatla Peninsula	EU938412	FJ004239
11	UAM 258	Alaska: Dall Island	EU938413	
12	GBP 550–55	Canada: Alberta: Along Hwy 881, vicinity of Imperial Mills	EU938414	FJ004240–43
13	GBP 564, 565	Canada: Alberta: Long Lake, 19.5 km W of Meanook Biological Research Station		FJ004244–45
14	GBP 556–59, 562, 647, 652, 653, 656, 683	Canada: Alberta: University of Alberta, Meanook Biological Research Station (and vicinity), ca. 12 km SW of Athabasca	EU938415	FJ004246–52
15	GBP 271–73, 275, 276	Canada: Alberta: ca. 4 km NW of west entrance gate to Elk Island National Park, ca. 40 km NE of Edmonton	EU938416	FJ004253–55
16	GBP 541, 542, 544, 546, 547, 549	Canada: Alberta: vicinity of Lodgepole	EU938417	FJ004256–59
17	GBP 319	Montana: Glacier County: Glacier National Park: Hanging Gardens, near Logan Pass	EU938418	FJ004260
18	GBP 161	Montana: Glacier-Flathead County Line: Glacier National Park, Three Bear Lake	EU938419	FJ004261
19	GBP 169	Montana: Flathead County: Lost Trail National Wildlife Refuge, north of Marion	EU938420	FJ004262
20	GBP 118	Washington: Skamania County: Mt. St. Helens National Volcanic Monument	EU938421	FJ004263
21	GBP 768	Wyoming: Teton County: Buffalo Fork River, 13km E of Moran Junction	EU938422	
22	MVZ 142827	Oregon: Lake County: ca. 24 km NE of Lakeview	EU938423	FJ004264
23	GBP 374	California: Sacramento County: 10.3 km east of Hwy 99 on Elverta Rd.	EU938424	
24	GBP 891	California: Napa County: Quail Ridge Ecological Preserve	EU938425	
25	GBP 584	California: Marin County Novato	EU938426	
26	GBP 595	California: Alameda-San Joaquin County Line: Corral Hollow	EU938427	FJ004265
27	GBP 501	California: Santa Clara County: San Antonio Valley	EU938428	

28	GBP 341	California : Santa Clara County: San Jose: Evergreen Valley College Campus	EU938429	
29	GBP 356	California: Santa Clara County: San Jose: Silver Creek	EU938430	
30	GBP 443	California: Fresno County: Lyon Rd. at Jensen Rd.	EU938431	
31	GBP 338	California: Fresno County: Panoche Rd at Interstate 5	EU938432	
32	GBP 429	California: Tulare County: ca. 8 km W of Visalia	EU938433	
33	GBP 608	California: Santa Barbara County: ca. 12 km NE of Los Olivos	EU938434	FJ004266
34	GBP 314	California: San Bernadino County: Fawnskin: Dana Point	EU938435	
35	GBP 306	California: San Bernadino County: Jenks Lake	EU938436	
36	GBP 401	California: Riverside County: Lake Hemet	EU938437	
37	GBP 376	California: Riverside County: Cleveland Forest Rd.	EU938438	
38	MVZ 145227	California: San Diego County: ca. 11.2 km SW of Warner Springs	EU938439	FJ004267
39	SDSNH 72548	California: San Diego County: Pamo Valley, N of Ramona	EU938440	
40	SDSNH 72587	California: San Diego County: Laguna Recreation Area: Burnt Rancheria Campground (# 13)	EU938441	
41	GBP 630	California: San Diego County: Descanso, Sweetwater River	EU938442	
42	GBP 639	California: San Diego County: Pine Valley: Pine Valley Creek	EU938443	FJ004268
43	SDSNH 72569	California: San Diego County: Anza Borrego Desert State Park: Bow Willow Campground	EU938444	
Bufo hemiophrys				
	MVZ 137738	Minnesota: Traverse County: vicinity of Wheaton		FJ004269
	UA-CT 157, 158	Canada: Alberta: 23 km NE of Imperial Mills		FJ004270–71
	UA-CT 177, 969	Canada: Alberta: 21 km NW of Imperial Mills		FJ004272
Bufo americanus				
	TNHC 62701	Kansas: Cherokee County: Riverton	EU938446	FJ004273
Bufo punctatus				
	TNHC 58788	Texas: Gillespie County: 3.75 km N of Willow City	EU938445	

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