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Molecular systematics, hybridization, and phylogeography of the *Bufo americanus* complex in Eastern North America

Susan E. Masta,[a,*] Brian K. Sullivan,[b] Trip Lamb,[c] and Eric J. Routman[a]

[a] *Department of Biology, San Francisco State University, 1600 Holloway Ave, San Francisco, CA 94132, USA*
[b] *Department of Life Sciences, Arizona State University West, P.O. Box 37100, Phoenix, AZ 85069, USA*
[c] *Department of Biology, East Carolina University, Greenville, NC 27858, USA*

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Abstract

We reconstruct phylogenetic relationships among a well-studied group of toads and find relationships that differ greatly from the current taxonomic understanding. We use mitochondrial sequences encoding ND1, $tRNA^{Leu(UUR)}$, and part of 16S to infer relationships among members of the *Bufo americanus* complex. Focusing on the four taxa that historically have been most problematic due to morphological similarity and hybridization in sympatry, we sample 150 individuals from multiple populations across each species' geographic range. Our evidence conflicts with previous taxonomic hypotheses that were based on ability to hybridize, geographic distribution, and call variation. First, sequences from *B. fowleri* do not comprise the sister clade to sequences of *B. woodhousii*; therefore the previous classifications of *B. fowleri* as sister species to, or eastern subspecies of, *B. woodhousii* are both called into question. Second, sequences from *B. americanus* are more closely related to those of *B. woodhousii* than to those of *B. terrestris*, indicating that similar advertisement call characteristics evolved independently. Third, sequences of *B. fowleri* are paraphyletic, with sequences of *B. terrestris* embedded within. Lastly, sequences from *B. fowleri* cluster into three distinct mitochondrial clades, with some divergences corresponding to greater than 2 mya. These clades are somewhat geographically structured, suggesting divergence in allopatry during the Pleistocene. These mitochondrial divergences are not accompanied by known phenotypic differences, however, suggesting either evolutionary stasis in morphology and behavior, cryptic phenotypic evolution, or that hybridization in secondary contact has homogenized phenotypic differences that may have arisen in allopatry.

Keywords: *Bufo americanus* group; *Bufo* systematics; Hybridization; Geographic variation; Call evolution; mtDNA

1. Introduction

The taxonomic relationships and number of species in the *Bufo americanus* group have been widely debated, particularly with regard to the status of *B. fowleri* (e.g., Cope, 1889; Girard, 1854; Myers, 1927, 1931; Sanders, 1987). The group is thought to consist primarily of *B. americanus*, *B. terrestris*, *B. microscaphus*, *B. hemiophrys*, *B. houstonensis*, *B. woodhousii*, and *B. fowleri*, with relict populations also described for *B. hemiophrys* and *B. microscaphus* (Porter, 1968; Smith et al., 1998; Gergus, 1998). Although *B. fowleri* was formerly considered an eastern subspecies of *B. woodhousii*, recent work by Sullivan et al. (1996) found distinct call differences between *B. woodhousii fowleri* and *B. woodhousii woodhousii*. Thus, many authors now recognize *B. fowleri* as a distinct species.

Blair's (1963) detailed studies of hybridization, mating call variation, and geographic distributions among North American *Bufo* led him to postulate phylogenetic relationships among these toads. Subsequent work on albumin immunological genetic distances within *Bufo*, however, demonstrated that ability to hybridize does not necessarily correlate with protein divergence (Wilson et al., 1974) as Blair had assumed. Proteins appear to evolve at a steady, clock-like rate in anurans (Wallace et al., 1971) and are therefore suitable for making phylogenetic inferences, but ability to hybridize may not reliably indicate phylogenetic relationships. Within the

* Corresponding author. Fax: +1-415-338-2295.
E-mail address: smasta@sfsu.edu (S.E. Masta).

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B. americanus complex, Blair (1963) proposed relationships based upon similarities in advertisement calls. He proposed that *B. americanus* and *B. terrestris* are sister taxa, *B. hemiophrys* and *B. microscaphus* are sister taxa, and *B. woodhousii* diverged earliest, with *B. woodhousii fowleri* an eastern race of *B. woodhousii*. Although hybridization ability is no longer accepted as a good character for phylogeny reconstruction, Blair's (1963) suggestion that advertisement call types are conserved characters in evolution, and therefore good phylogenetic characters, has not been widely disputed.

Toads in the genus *Bufo* are able to hybridize with distantly related congeners (reviewed in Blair, 1972). Hybridization is especially prevalent among members of the *B. americanus* complex, for which numerous studies have documented zones of hybridization and examined possible isolating mechanisms between species (e.g., Blair, 1941; Cory and Manion, 1955; Brown, 1970b; Green, 1996; Meacham, 1962; Thornton, 1955; Volpe, 1959).

Interspecific hybridization can confound our attempts to infer phylogenetic relationships. This is especially the case when hybridizing species occur sympatrically over large portions of their ranges. Hybrid individuals have often been discerned when they show characteristics intermediate between parental types; however, they may instead possess a combination of traits that are characteristic of each of their parents (e.g., McDade, 1990). In some interspecific hybrids of *Bufo* toads, certain traits, such as mating calls (Green, 1982) and morphology (Meacham, 1962), appear intermediate between parental species. However, amphibian hybrids are not always morphologically intermediate, but may instead be indistinguishable in appearance from one of their parental types and discernible only by analyzing genetic markers (e.g., Lamb and Avise, 1987; Wake et al., 1980).

Molecular markers have greatly aided the study of hybridization, because they provide more characters than morphology alone with which to discern hybrid individuals. They have helped uncover previously unknown cases of hybridization and have helped measure the extent of hybrid zones and introgression of genes between species (e.g., Ferris et al., 1983; Lehman et al., 1991; Rieseberg et al., 1990). Mitochondrial DNA appears to be maternally inherited and non-recombining in most metazoans (reviewed in Moritz et al., 1987), and thus, is ideal for inferring the maternal genealogy of animal species that are known to hybridize. Because mitochondrial genes do not recombine, interspecific hybridization will not affect the genealogical reconstruction of mitochondrial sequences and their relationships can be depicted by bifurcating genealogies.

A well-resolved modern cladistic hypothesis for the *B. americanus* group has not been put forward, probably for two major reasons. First, hybridization often makes it difficult to reliably assign individuals to a particular taxon. Second, few morphological characters safely distinguish among species in this group. The task of accurate assignment to species is simplified for taxa with largely allopatric populations. For example, *B. microscaphus* shows minimal overlap with the ranges of other members of the *B. americanus* complex, making interspecific hybridization unlikely, except with *B. wood-*

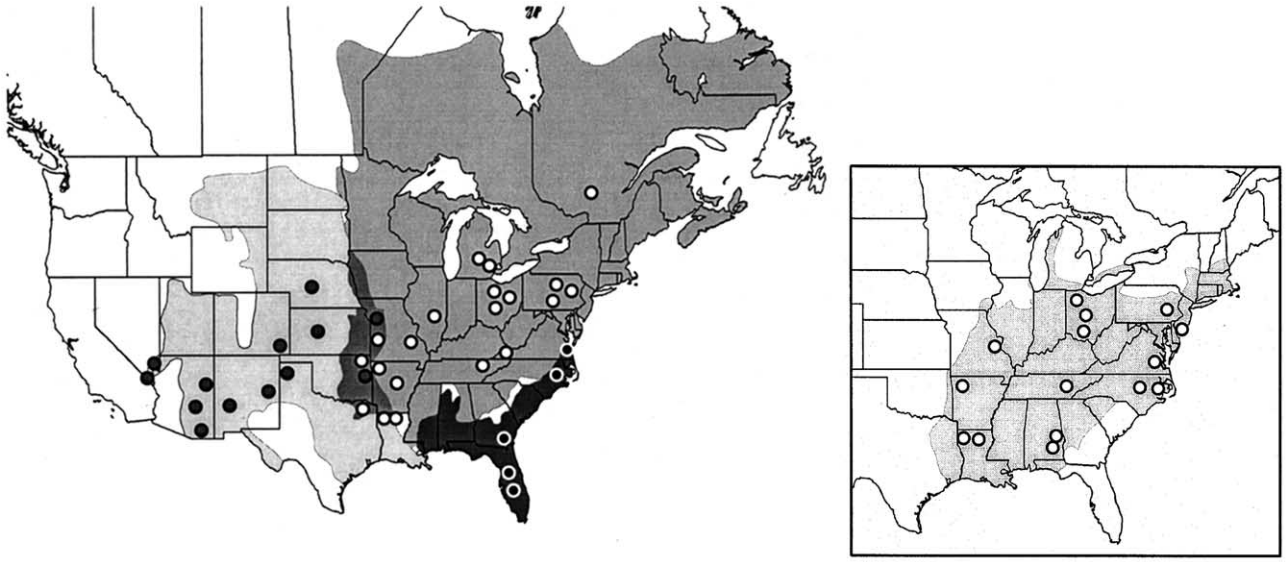

Fig. 1. Geographic distributions of *B. americanus*, *B. terrestris*, and *B. woodhousii* (left) and *B. fowleri* (inset) in the US and Canada, modified from Conant and Collins (1991). The lightest shade of gray indicates the range of *B. woodhousii*, the next darker shade of gray the range of *B. americanus*, with darker shading indicating the area of sympatry between these species. The darkest shade indicates the range of *B. terrestris*. The shaded area in the inset indicates the range of *B. fowleri*. Circles indicate approximate collection locations of specimens.

housii in narrow zones of sympatry in Arizona (Blair, 1955; Sullivan, 1985). However, in the eastern US, four members of the complex occur sympatrically over large areas (Fig. 1). The range of *B. fowleri* overlaps with those of *B. woodhousii* in the central US, *B. terrestris* in the southeastern US, and *B. americanus* over nearly their entire range.

Here, we seek to determine the molecular phylogenetic relationships among these four problematic, largely sympatric, members of the *B. americanus* group from eastern North America. Because these toads are known to hybridize, we could not simply adopt the approach typically used in molecular systematics of sequencing one to a few individuals per species. Instead, we first built gene trees from mitochondrial sequences from specimens of *B. americanus*, *B. terrestris*, and *B. woodhousii*, which were fully allopatric with one another and with *B. fowleri*. Determining relationships initially among these three taxa alone enabled us to avoid being confounded by historical hybridization and introgression of mitochondrial haplotypes. Then, we reconstructed gene trees that included *B. fowleri* and sympatric individuals of the other three species, to determine the relationships of *B. fowleri* to these members of the *B. americanus* complex. Both to sample the genetic diversity within a species and to minimize the problem of cryptic hybrid individuals in sympatry, we collected numerous specimens of *B. americanus*, *B. terrestris*, *B. woodhousii*, and *B. fowleri* across their respective ranges.

2. Materials and methods

2.1. Specimen information

One hundred forty-six toads were collected from across the US, covering much of the geographic range of each taxon (Fig. 1). Tissue was removed and museum voucher specimens were prepared for most animals. Additional tissues were obtained from the Museum of Vertebrate Zoology at the University of California at Berkeley.

While individuals collected in locations allopatric with all other members of the *B. americanus* group were easily assigned to species, in regions of sympatry systematic scoring of morphological characters was used to make species assignments, as we recognized the potential for encountering hybrid individuals in our sampling effort. We thus employed a hybrid index to score specimens of *B. americanus* and *B. fowleri* collected in sympatry. This was done using four of the characters described by Jones (1973) and Conant and Collins (1991): cranial crest contact with parotid glands, size of tibial warts, presence of ventral spotting, and number of warts per dorsal spot. Toads received pure parental classification if they showed at least three of the four defining characters, as suggested by Conant and Collins (1991), or as a hybrid if they possessed less than three species-specific characters. *B. fowleri* also hybridizes with *B. terrestris*; however, no morphological characters can reliably be used to identify hybrid offspring, as the only unique feature of *B. terrestris* (knobs on the cranial crests) is not typically present in hybrid crosses (Volpe, 1959). Therefore, specimens collected from areas where these taxa are sympatric were scored as *B. terrestris* if they had pronounced cranial crest knobs, consistent with those described by Volpe (1959, Fig. 3). In areas of sympatry between *B. fowleri* and *B. woodhousii*, toads were classified as *B. fowleri* if they showed three or more warts per dorsal spot. Table 1 lists specimen identifications, field collection numbers, and collection locations.

2.2. Molecular techniques and sequence accuracy

Genomic DNA was extracted from muscle, toes, liver or blood using a modification of a previously described salt extraction technique (Hillis et al., 1996). DNA isolated from blood and liver was subsequently cleaned with phenol and chloroform extractions, following typical protocols. Mitochondrial DNA genes were amplified by the polymerase chain reaction, with denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min 15 s. Amplification primers were designed specifically for *Bufo* to amplify a region encompassing about 100 bp of the 3′ end of 16S, all of $tRNA^{Leu(UUR)}$, NADH dehydrogenase subunit 1 (ND1), $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, and about 90 bp NADH dehydrogenase subunit 2 (ND2). This was done using previously published primers that amplify other vertebrates (Palumbi et al., 1991; Kumazawa and Nishida, 1993) for initial PCR, gel purifying and sequencing the PCR products, then designing internal primers that were conserved across *Bufo*. For sequencing, two primers were used, the PCR primer located in the 16S region and an internal primer that binds to the $tRNA^{Ile}$-ND1 genes (see Table 2). Sequencing reactions were performed using dye-labeled terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit), using the cycling conditions recommended by the manufacturer. Sequencing reactions were analyzed on an ABI Prism 377 automated DNA sequence analysis machine in the Conservation Genetics Lab at San Francisco State University.

All chromatograms were first checked by eye for base call accuracy and then aligned individually with the opposite strand from the same individual using the program Sequencer, and bases were reexamined for sequence agreement. Finally, all sequences were aligned and compared with all other *Bufo* sequences, with each variable site again scored for accuracy of base calls. Base ambiguities were coded with IUPAC symbols. This

Table 1
Specimen information

Tree code #	Species designation	Morphology score	State	County/Parish[a]	Collection #	GenBank #
1	*B. americanus charlesmithi*	3A/1F	LA	Caddo	BKS1088	AF462469
2	*B. americanus charlesmithi*	3A/1F	LA	Caddo	BKS1089	AF462470
3	*B. americanus charlesmithi*	3A/1F	LA	Caddo	BKS1091	same as SEM350
4	*B. americanus charlesmithi*	3A/1F	LA	Caddo	BKS1092	AF462471
5	*B. americanus charlesmithi*	3A/1F	LA	Ouachita	BKS1098	same as BKS1102
6	*B. americanus charlesmithi*	4A	LA	Ouachita	BKS1100	AF462472
7	*B. americanus charlesmithi*	4A	LA	Ouachita	BKS1103	same as BKS1100
8	*B. americanus charlesmithi*	3A/1F	AR	Hot Springs	BKS1142	AF462473
9	*B. americanus charlesmithi*	3.5A/.5F	AR	Hot Springs	BKS1145	same as BKS1140
10	*B. americanus charlesmith*i	3A/1F	AR	Madison	BKS1147	AF462473
11	*B. americanus charlesmithi*	3A/1F	AR	Madison	BKS1148	same as SEM370
12	*B. americanus charlesmithi*	3.5A/.5F	AR	Madison	BKS1149	AF462475
13	*B. americanus charlesmithi*		MO	Laclede	EJR6294	AF462476
14	*B. americanus charlesmithi*	4A	OK	Muskogee	SEM345	AF462468
15	*B. americanus charlesmithi*	3.5A/.5F	MO	St. Clair	SEM431	AF462477
16	*B. americanus charlesmithi*	4A	TX	Fannin	SEM319	AF462467
17	*B. americanus charlesmithi*	4A	TX	Fannin	SEM320	same as SEM319
18	*B. americanus charlesmithi*	3A/1W	OK	Muskogee	SEM370	same as SEM345
19	*Bufo americanus*		MI	Washtenaw	SEM2070	AF462478
20	*Bufo americanus*	4A	IL	Madison	BKS1130	AF462481
21	*Bufo americanus*	3A/1F	PA	Union	BKS1136	AF462482
22	*Bufo americanus*	3.5A/.5F	PA	Bucks	SEM2266	AF462484
23	*Bufo americanus*	4A	PA	Bucks	SEM2267	same as SEM2266
24	*Bufo americanus*	3.5A/.5F	PA	Columbia	SEM2268	AF462485
25	*Bufo americanus*	3.5A/.5F	PA	Columbia	SEM2269	same as SEM2266
26	*Bufo americanus*	3.5A/.5F	PA	York	SEM2270	same as SEM2266
27	*Bufo americanus*	4A	PA	Lancaster	SEM2271	AF462486
28	*Bufo americanus*	3A/1F	PA	Lancaster	SEM2272	same as SEM2266
29	*Bufo americanus*	3.5A/.5F	PA	Lehigh	SEM2278	AF462487
30	*Bufo americanus*	3.5A/.5F	PA	Lehigh	SEM2280	AF462488
31	*Bufo americanus*	3A/1F	PA	Lehigh	SEM2281	AF462489
32	*Bufo americanus*	3A/1F	PA	Lehigh	SEM2282	AF462490
33	*Bufo americanus*	4A	OH	Athens	SEM2288	same as SEM2266
34	*Bufo americanus*	4A	OH	Green	SEM2289	AF462491
35	*Bufo americanus*	3.5A/.5F	OH	Licking	SEM2290	AF462492
36	*Bufo americanus*	3.5A/.5F	OH	Licking	SEM2291	same as SEM2290
37	*Bufo americanus*	3.5A/.5F	OH	Perry	SEM2292	AF462493
38	*Bufo americanus*	4A	OH	Perry	SEM2293	AF462494
39	*Bufo americanus*	3.5A/.5F	OH	Lawrence	SEM2295	AF462495
40	*Bufo americanus*	4A	OH	Lawrence	SEM2296	AF462496
41	*Bufo americanus*	3.5A/.5F	OH	Butler	SEM2297	AF462497
42	*Bufo americanus*	3A/1F	OH	Butler	SEM2298	same as SEM2296
43	*Bufo americanus*	3.5A/.5F	OH	Hamilton	SEM2299	AF462498
44	*Bufo americanus*	4A	OH	Pike	SEM2300	same as SEM2296
45	*B. americanus/fowleri* hybrid	2A/2F	OH	Lucas	SEM2304	same as SEM2289
46	*B. americanus/fowleri* hybrid	2A/2F	OH	Union	SEM2309	AF462499
47	*Bufo americanus*	3A/1F	OH	Union	SEM2310	same as SEM2296
48	*Bufo americanus*	3A/1F	TN	Cumberland	SEM2318	AF462500
49	*Bufo americanus*	4A	VA	Scott	SEM2319	AF462501
50	*Bufo americanus*		QB	Terrebone	MVZ137728	AF462483
51	*Bufo americanus*		MI	Wayne	SEM2071	AF462479
52	*Bufo americanus*	4A	MI	Wayne	SEM2072	AF462480
53	*B. americanus/fowleri* hybrid	2A/2F	LA	Ouachita	BKS1101	AF462461
54	*B. americanus/fowleri* hybrid	2.5A/1.5F	LA	Ouachita	BKS1102	AF462462
55	*B. americanus/fowleri* hybrid	2A/2F	AL	Macon	BKS1128	AF462463
56	*B. americanus/fowleri* hybrid	2.5A/1.5F	AR	Hot Springs	BKS1139	AF462464
57	*B. americanus/fowleri* hybrid	2.5A/1.5F	AR	Hot Springs	BKS1141	same as BKS1140
58	*B. americanus/fowleri* hybrid	2.5A/1.5F	PA	Lehigh	SEM2279	AF462466
59	*B. fowleri/americanus* hybrid	1.5A/2.5F	AR	Hot Springs	BKS1140	AF462465
60	*B. fowleri/americanus* hybrid	1.5A/2.5F	AR	Hot Springs	BKS1143	same as SEM350

Table 1 (*continued*)

Tree code #	Species designation	Morphology score	State	County/Parish[a]	Collection #	GenBank #
61	*B. fowleri*/*americanus* hybrid	2A/2F	AR	Hot Springs	BKS1144	same as BKS1140
62	*B. fowleri*/*woodhousii* hybrid	1.5W/2.5F	IL	Madison	BKS1131	same as BKS1146
63	*Bufo fowleri*	0T	NC	Pitt	ACL488	same as SEM2260
64	*Bufo fowleri*	1A/3F	LA	Caddo	BKS1090	same as BKS1089
65	*Bufo fowleri*	1A/3F	LA	Ouachita	BKS1099	AF462506
66	*Bufo fowleri*	.5A/3.5F	AL	Lee	BKS1122	AF462507
67	*Bufo fowleri*	4F	AL	Lee	BKS1123	same as BKS1122
68	*Bufo fowleri*	4F	AL	Macon	BKS1126	AF462508
69	*Bufo fowleri*	1A/4F	AL	Macon	BKS1127	same as BKS1122
70	*Bufo fowleri*	4F	AL	Macon	BKS1129	AF462509
71	*Bufo fowleri*	.5A/3.5F	MO	St. Charles	BKS1133	AF462510
72	*Bufo fowleri*	4F	NJ	Cape May	SEM2250	AF462511
73	*Bufo fowleri*	4F	NJ	Cape May	SEM2251	AF462512
74	*Bufo fowleri*	4F	VA	New Kent	SEM2258	AF462513
75	*Bufo fowleri*	4F	VA	New Kent	SEM2259	AF462514
76	*Bufo fowleri*	3.5F	VA	New Kent	SEM2260	AF462515
77	*Bufo fowleri*	4F	VA	New Kent	SEM2261	AF462516
78	*Bufo fowleri*	4F	VA	New Kent	SEM2262	same as SEM2261
79	*Bufo fowleri*	4F	PA	Lehigh	SEM2283	AF462517
80	*Bufo fowleri*	4F	PA	Lehigh	SEM2284	same as SEM2258
81	*Bufo fowleri*	.5A/3.5F	PA	Lehigh	SEM2285	same as SEM2258
82	*Bufo fowleri*	4F	PA	Lehigh	SEM2286	same as SEM2258
83	*Bufo fowleri*	4F	PA	Lehigh	SEM2287	same as SEM2283
84	*Bufo fowleri*	4F	OH	Wood	SEM2301	AF462518
85	*Bufo fowleri*	4F	OH	Ross	SEM2305	same as SEM2258
86	*Bufo fowleri*	4F	OH	Ross	SEM2306	AF462519
87	*Bufo fowleri*	4F	OH	Union	SEM2307	AF462520
88	*Bufo fowleri*	4F	OH	Union	SEM2308	same as SEM2307
89	*Bufo fowleri*	0T	NC	Pitt	ACL502	same as SEM2260
90	*Bufo fowleri*	0T	NC	Pitt	ACL503	same as SEM2260
91	*Bufo fowleri*	0T	NC	Pitt	ACL504	same as SEM2260
92	*Bufo fowleri*	.5A/3.5F	TN	Cumberland	SEM2314	AF462521
93	*Bufo fowleri*	4F	TN	Cumberland	SEM2315	AF462522
94	*Bufo fowleri*	4F	TN	Cumberland	SEM2316	AF462523
95	*Bufo fowleri*	4F	TN	Cumberland	SEM2317	same as SEM2316
96	*Bufo fowleri*	0T	NC	Wake	SEM2320	AF462524
97	*Bufo fowleri*	0T	NC	Wake	ALB10446	same as SEM2260
98	*Bufo fowleri*	0T	NC	Wake	SEM2323	AF462525
99	*Bufo fowleri*	0T	NC	Wake	SEM2324	AF462526
100	*Bufo fowleri*	.5A/3.5F	AR	Madison	BKS1146	AF462505
101	*Bufo fowleri*	4F	MO	St. Charles	BKS1132	same as SEM350
102	*Bufo terrestris*	3T	NC	Hertford	ACL456	AF462532
103	*Bufo terrestris*	4T	NC	Hertford	ACL457	AF462533
104	*Bufo terrestris*	4T	FL	Marion	MVZ137326	AF462529
105	*Bufo terrestris*	4T	FL	Charlotte	MVZ223380	AF462531
106	*Bufo terrestris*	4T	GA	Clinch	MVZ150034	AF462530
107	*Bufo terrestris*	3T	VA	City of VA Beach	SEM2252	AF462534
108	*Bufo terrestris*	3T	VA	City of VA Beach	SEM2253	same as SEM2266
109	*Bufo terrestris*	3T	VA	City of VA Beach	SEM2255	same as SEM2278
110	*Bufo terrestris* hybrid	2T	VA	City of VA Beach	SEM2254	AF462535
111	*Bufo terrestris* hybrid	2T	VA	City of VA Beach	SEM2256	same as SEM2266
112	*Bufo terrestris* hybrid	2T	VA	City of VA Beach	SEM2257	AF462536
113	*Bufo woodhousii*		NV	Nye	EJR6549	AF462537
114	*Bufo woodhousii*		NV	Clark	EJR6584	same as EJR6549
115	*Bufo woodhousii*		AZ	Navajo	EJR7055	AF462538
116	*Bufo woodhousii*		AZ	Navajo	EJR7056	AF462539
117	*Bufo woodhousii*		AZ	Navajo	EJR7057	same as SEM2101
118	*Bufo woodhousii*		AZ	Gila	SEM102	AF462540
119	*Bufo woodhousii*		AZ	Gila	SEM103	same as SEM102
120	*Bufo woodhousii*		AZ	Gila	SEM104	AF462541
121	*Bufo woodhousii*		AZ	Gila	SEM105	same as SEM104
122	*Bufo woodhousii*		NM	Socorro	SEM145	same as SEM104
123	*Bufo woodhousii*		NM	Socorro	SEM146	same as SEM104
124	*Bufo woodhousii*		NM	Socorro	SEM147	same as SEM104

Table 1 (*continued*)

Tree code #	Species designation	Morphology score	State	County/Parish[a]	Collection #	GenBank #
125	*Bufo woodhousii*		NM	Socorro	SEM148	AF462542
126	*Bufo woodhousii*		KS	Barton	SEM452	same as SEM350
127	*Bufo woodhousii*		KS	Barton	SEM455	AF462545
128	*Bufo woodhousii*		TX	Hartley	SEM891	same as SEM350
129	*Bufo woodhousii*		CO	Prowers	SEM1756	AF462547
130	*Bufo woodhousii*		CO	Prowers	SEM1757	same as SEM350
131	*Bufo woodhousii*		AZ	Cochise	SEM2101	same as EJR7055
132	*Bufo woodhousii*		AZ	Cochise	SEM2102	same as SEM2101
133	*Bufo woodhousii*		AZ	Cochise	SEM2103	AF462548
134	*Bufo woodhousii*		AZ	Cochise	SEM2114	AF462549
135	*Bufo woodhousii*		OK	Muskogee	SEM350	same as BKS1139
136	*Bufo woodhousii*		OK	Muskogee	SEM351	same as SEM350
137	*Bufo woodhousii*		OK	Muskogee	SEM353	same as SEM350
138	*Bufo woodhousii*		MO	Saline	SEM418	AF462543
139	*Bufo woodhousii*		MO	Saline	SEM419	same as SEM350
140	*Bufo woodhousii*		MO	Saline	SEM420	same as SEM418
141	*Bufo woodhousii*		MO	Saline	SEM421	AF462544
142	*Bufo woodhousii*		TX	Hartley	SEM913	same as SEM350
143	*Bufo woodhousii*		NM	Chaves	SEM1431	AF462546
144	*Bufo woodhousii*		NM	Chaves	SEM1432	same as SEM350
145	*Bufo microscaphus*		AZ	Yavapai	BKS1081	AF462527
146	*Bufo microscaphus*		AZ	Yavapai	BKS1082	AF462528
147	*Bufo cognatus*		CA	San Bernardino	EJR7103	AF462504
148	*Bufo cognatus*		TX	Parmer	EJR4797	AF462502
149	*Bufo cognatus*		KS	Harper	EJR5005	AF462503
150	*Bufo cognatus*		TX	Hartley	SEM904	same as EJR7103

[a] Additional collection and specimen information is available from the first author upon request.

method allowed us to be confident that sequences were scored correctly. However, there have been numerous reports of mitochondrial gene copies transposed to the nucleus (e.g., Zhang and Hewitt, 1996). To ascertain that these *Bufo* sequences were not nuclear pseudogene copies, the protein-coding ND1 sequences were translated to amino acids using the program MacClade 4 (Maddison and Maddison, 2000) to look for the presence of stop codons or frameshifts. Additionally, the $tRNA^{Leu(UUR)}$ sequences were manually folded using the criterion of Kumazawa and Nishida (1993) to ascertain they had stable secondary structures.

2.3. Phylogenetic analyses

Final alignment of sequences was performed manually and identical haplotypes were merged using MacClade 4 (Maddison and Maddison, 2000). Sequences from *B. microscaphus*, *B. americanus*, *B. terrestris*, and *B. woodhousii* that were collected in locations allopatric with respect to all other members of the *B. americanus* group were first used to build gene trees to determine the interrelationships of their mitochondrial sequences. Sequences of all *B. fowleri* individuals were excluded from these initial analyses, as the geographic range of *B. fowleri* is broadly sympatric with that of *B. americanus*, *B. terrestris*, and *B. woodhousii*. Subsequently, sequences from all individuals from across their geographic ranges (including those of *B. fowleri*) were used to build a second set of gene trees.

Gene trees were constructed using PAUP*4.0b8 (Swofford, 2000), using a variety of tree-building strategies. For the set of allopatric taxa, parsimony trees were constructed using a branch-and-bound search. Five hundred nonparametric bootstrap replicates were per-

Table 2
Primer information

Name[a]	Gene location	*Xenopus* position[b]	Sequence
Bw16S-L	16S	4604	5′-ATTTTTTCTAGTACGAAAGGAC-3′
BwND2-H	ND2	6097	5′-ATGGCTAATGTGTTAATTTC-3′
B-Ile-H	$tRNA^{Ile}$-ND1	5779	5′-GCACGTTTCCATGAAATTGGTGG-3′

[a] H and L designate heavy and light strand primers, respectively.
[b] Position refers to first base of 5′ end of primer in the *X. laevis* mitochondrial genome (Roe et al., 1985).

formed to estimate branch support (Felsenstein, 1985). Maximum likelihood (ML) trees were sought by first obtaining a neighbor-joining tree using the HKY85 model of evolution (Hasegawa et al., 1985) and using that tree to estimate base frequencies, transition/transversion ratio, and gamma shape parameter. These parameter estimates were then used for an ML search, after which the above parameters were again estimated on the tree. This successive approximation iteration was conducted for 8 ML searches, following the suggestions of Swofford et al. (1996). This method recovered multiple trees of the same likelihood score and searches were considered complete when multiple trees with the same, lowest log likelihood score were found.

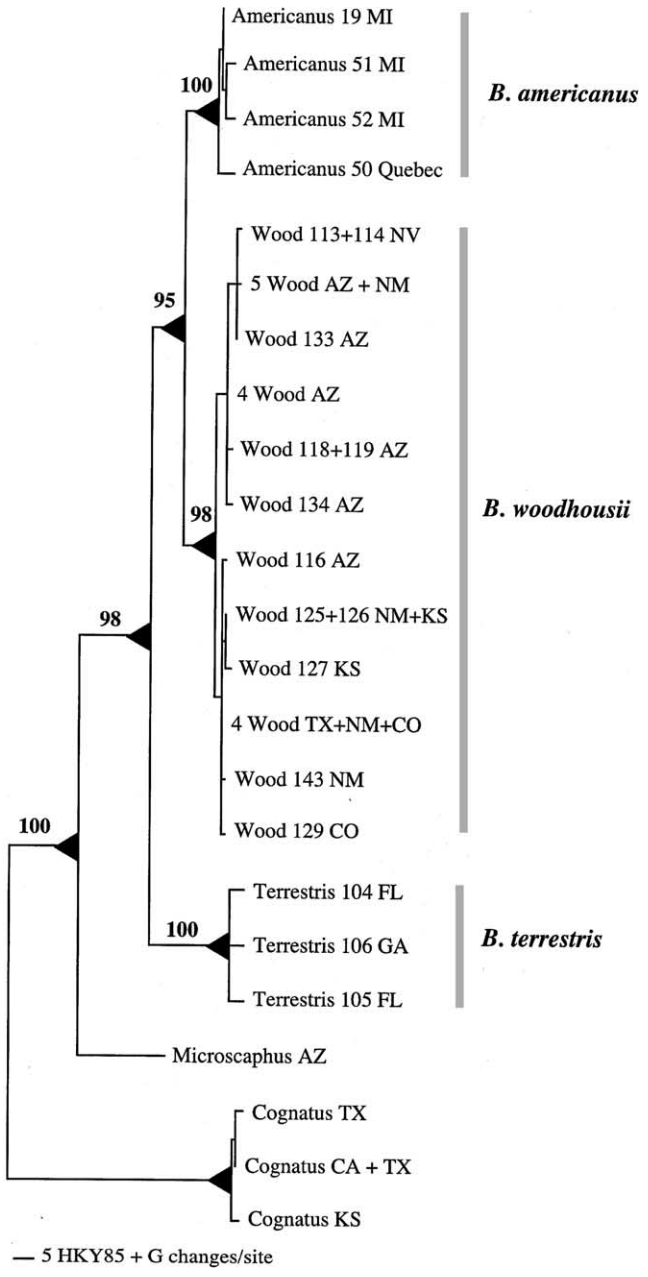

Fig. 2. Phylogenetic relationships of sequences of specimens collected from allopatric portions of ranges only. Shown is ML tree with the lowest −ln likelihood score, using the HKY+G model of evolution (−ln = 2549.97; ti/tv = 10.179; gamma shape parameter = 0.016). A black triangle at the base of a clade indicates the clade was recovered in all analyses (parsimony, neighbor-joining, and ML). Bootstrap values from parsimony analyses that are greater than 75% are shown above the branches. Taxa are labeled with the species name, followed by their identification number for the gene trees, followed by the state in which the animal was collected. Refer to Table 1 for further information. Identical haplotypes found among four or more individuals are coded only with the number of individuals that possessed the haplotype and the states in which they were collected.

For the full 150-specimen data set, parsimonious trees were sought with a heuristic search with 10 random addition sequences and TBR rearrangements (equal weighting, unordered states), holding a maximum of 5000 trees. One thousand bootstrap replicates were performed. Because a single heuristic search does not guarantee that the shortest trees will be found, a series of searches was conducted to search for islands of shorter trees (Maddison, 1991). We conducted 100 replicate searches, each limited to saving 50 trees of equal or shorter length than those found by the initial heuristic search. Gene trees were also constructed with neighbor-joining using the HKY85 model of evolution, with 1000 bootstrap replicates. ML trees were obtained as described above, except using the HKY85+G+I model of evolution and 10 random addition sequence heuristic searches. Four sequences of *B. cognatus* were used to root all trees, as this species has been shown to fall outside the *B. americanus* group (Graybeal, 1997; Maxson et al., 1981).

To estimate the amount of genetic divergence among clades that were recovered in the above analyses, we used a measure of population divergence as suggested by Nei (1987, Eq. 10.20). The average number of nucleotide substitutions between sequences in different clades was calculated with the program DnaSP (Rozas and Rozas, 1997), with a Jukes and Cantor (1969) correction.

3. Results

A total of 150 sequences of approximately 1110 bp was obtained, aligned, and deposited in GenBank (see Table 1). A single bp indel was found in the 16S sequences of all *B. cognatus*. No stop codons or indels were present in translated ND1 sequences and most changes occurred at the third position of codons. All $tRNA^{Leu(UUR)}$ sequences were highly conserved with respect to the sequence of the frog *Xenopus laevis* (Roe et al., 1985) and could be folded into stable secondary structures (not shown). Together, these results suggest that these sequences are authentic mitochondrial sequences and not pseudogenes that have been transposed to the nucleus.

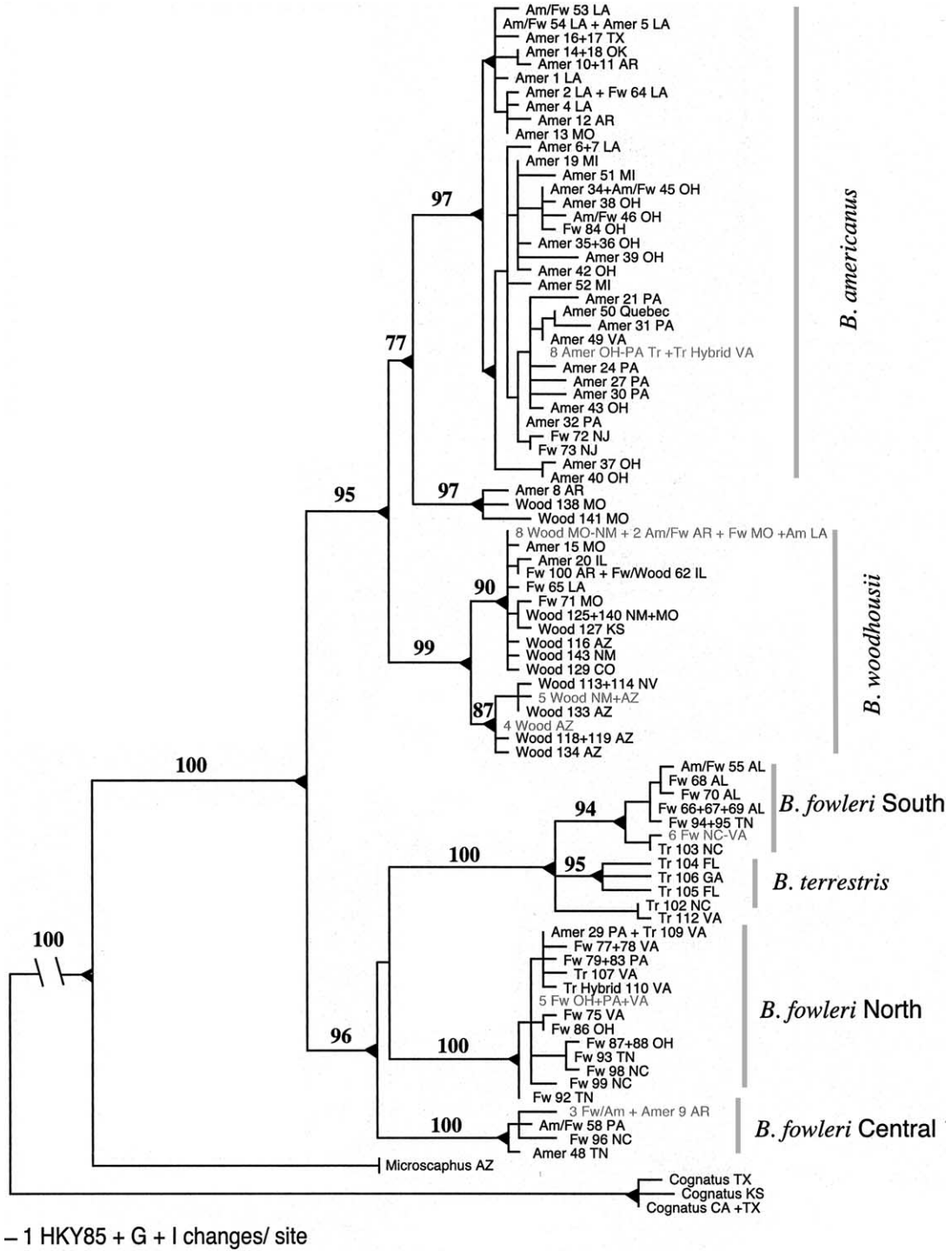

Fig. 3. Phylogenetic relationships of sequences of all specimens, sympatric and allopatric, including those of *B. fowleri*. Shown is ML tree with the lowest −ln likelihood score, using the HKY+G+I model of evolution (−ln = 3573.06; ti/tv = 11.06; gamma shape parameter = 0.708; % invariable sites = 0.549). A black triangle at the base of a clade indicates the clade was recovered in all analyses (parsimony, 100 parsimony island searches, neighbor-joining, and ML). Bootstrap values from parsimony analyses that are greater than 75% are shown above the branches. Taxa are labeled with the species name, followed by their identification number for the gene trees, followed by the state in which the animal was collected. Refer to Table 1 for further information. Identical haplotypes found among four or more individuals are colored gray and coded only with the number of individuals that possessed the haplotype and the state in which they were collected.

Sixty-one individuals possessed sequences identical to other individuals, yielding a total of 89 haplotypes. Two haplotypes were especially frequent and widespread: one found in nine individuals of *B. woodhousii* from across much of its central US range and one found in six individuals of *B. americanus* from the northern US.

All methods of tree construction resulted in trees that were highly concordant with one another; therefore only ML trees are shown. Gene trees of sequences from allopatric specimens show clearly distinct clades, with high bootstrap support (Fig. 2). Sequences from *B. microscaphus* form the sister group to those of the other *B. americanus* complex members. Sequences from *B. americanus* comprise a clade that is the sister clade to those of *B. woodhousii*. Finally, sequences from *B. americanus* and *B. woodhousii* together form the sister clade to sequences from *B. terrestris*.

Fig. 3 shows the 150-taxon tree, including sequences from *B. fowleri* and from sympatric individuals of the other three species. The *B. americanus* and *B. woodhousii* clades continue to show high bootstrap support and each contains within it distinct clades corresponding to geography. A clade consisting of sequences from three individuals from Missouri and Arkansas forms the sister clade to *B. americanus*, although bootstrap support is not high. Most of the sequences from *B. fowleri* cluster into three additional clades (named North, South, and Central in Fig. 3), each with high bootstrap support. One of these three is the sister clade to the clade composed of sequences from *B. terrestris*. The *B. terrestris* clade is nested among the three *B. fowleri* clades, making *B. fowleri* sequences paraphyletic. Sequences from *B. fowleri*, furthermore, do not comprise the sister clade to sequences from *B. woodhousii*; instead, *B. americanus* is more closely related to *B. woodhousii*. Among all species, sympatric individuals that were assigned species designations based on morphology occasionally possessed mtDNA sequences most closely related to those of other species.

The degree of genetic divergence between the clades of *B. woodhousii* and *B. americanus* is little more than half that between clades corresponding to *B. fowleri* from the north and south (Table 3). Using a molecular clock calibrated for *Bufo* mitochondrial ND1–ND2 genes with a rate of 0.69% change per lineage per million years (Macey et al., 1998), we estimate a divergence time between *B. woodhousii* and *B. americanus* of 1.3 mya and a divergence time of over 2 mya for *B. fowleri* from the northern versus the southern US.

4. Discussion

Three surprising results regarding the taxon known as "*B. fowleri*" emerge from this study's analysis of mitochondrial sequences. First, *B. fowleri* is composed of three separate clades, each well-supported and rather deeply diverged. Second, its sequences are paraphyletic, with one of its three clades being most closely related to *B. terrestris*. Third, *B. fowleri* sequences are not most closely related to those of *B. woodhousii*, of which it has traditionally been considered either a subspecies or the sister species.

Relationships among other members of the *B. americanus* group are also surprising in light of previous taxonomy. First, *B. microscaphus* sequences are sister to those of other members of the *B. americanus* complex, contrary to Blair's (1963) hypothesis that *B. woodhousii* diverged first from the group (see Fig. 4). Second, we do not find *B. americanus* and *B. terrestris* to be sister taxa, as suggested by Blair (1963). However, our findings are generally consistent with work based upon mitochondrial 12S, cytochrome oxidase I, and cytochrome *b* sequences from single individuals from the *B. americanus* group (Goebel, 1996). Although Goebel did not include *B. fowleri* in her analysis, she did include *B. hemiophrys* (a member of the *B. americanus* group not included in this study, as it shares no range overlap with *B. fowleri*). She found that *B. hemiophrys* was the sister taxon to *B. americanus*, forming a clade that was the sister taxon to *B. woodhousii* (Goebel, 1996). Therefore, her results are entirely consistent with our own.

4.1. Geographic variation

Bufo woodhousii shows two distinct mtDNA clades, one of which is localized in the southwestern US and is largely concordant with the range of *B. woodhousii australis*, a subspecies described by Shannon and Lowe (1955).

Bufo americanus charlesmithi, a subspecies of *B. americanus* in the southwestern part of the species' range, is found in Arkansas, Oklahoma, and portions of adjacent states (see Fig. 1). Individuals from Louisiana, Missouri, eastern Texas, and Oklahoma possessed se-

Table 3
Average number of nucleotide substitutions between clades[a]

	1	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)
1. *B. americanus*	—	1.8	3.96	3.48	2.98	3.23
2. *B. woodhousii*		—	3.93	3.78	2.89	3.39
3. *B. terrestris*			—	1.62	2.99	3.59
4. *B. fowleri* South				—	2.99	3.46
5. *B. fowleri* North					—	2.36
6. *B. fowleri* Central						—

[a] Per 100 sites.

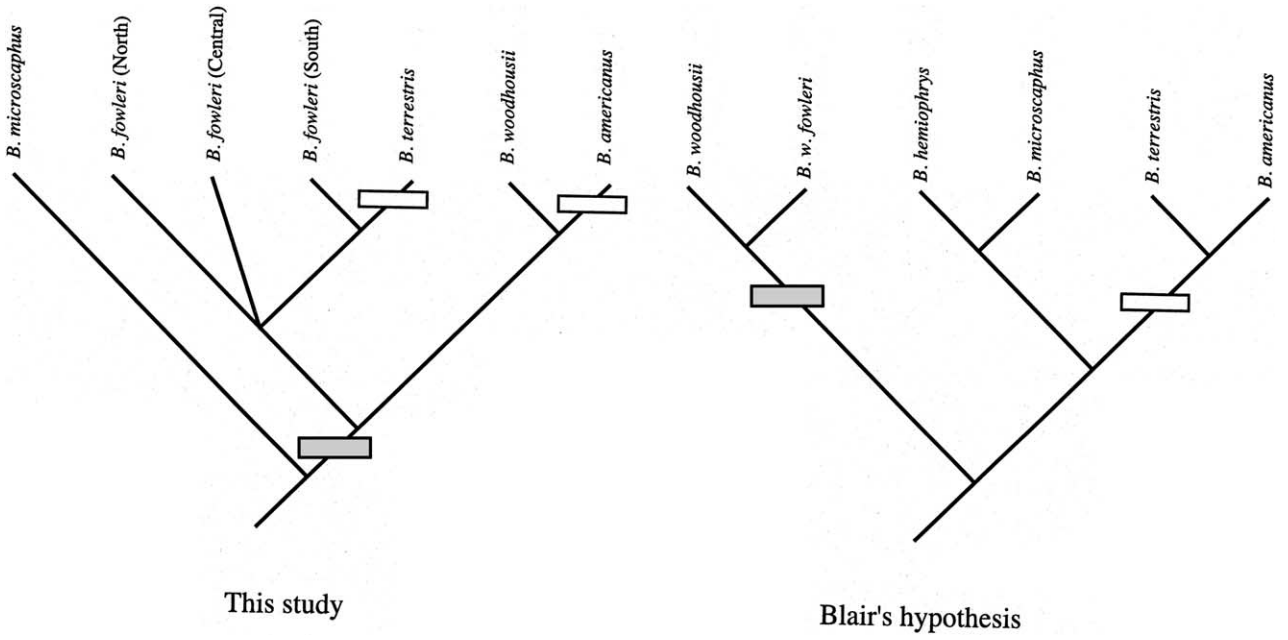

Fig. 4. Schematic drawings of the phylogenetic relationships recovered in this study (left) and Blair's (1963) hypothesis of relationships among *B. americanus* group members, based on call similarity (right). Advertisement call duration is mapped onto the trees, with long calls represented by unfilled rectangles and short calls by gray rectangles (see text for details).

quences that formed a clade in all analyses, although parsimony analyses did not yield high bootstrap support.

Bufo fowleri and associated hybrids have sequences that form three clades (Figs. 3 and 5). Thirteen *B. fowleri* individuals collected from the southeastern US, from Alabama through southeastern Virginia (the South clade), have haplotypes most closely related to sequences from *B. terrestris*. Another clade (the North clade) contains sequences from 17 *B. fowleri* individuals collected from central Tennessee north through Ohio and Pennsylvania.

The third (Central) clade is less geographically cohesive, consisting of individuals collected from four widely separated sites across the center of the range of *B. fowleri*. These individuals appear primarily to be morphological hybrids between *B. americanus* and *B. fowleri*. While one might at first consider the possibility of paraphyly of *B. americanus*, a more parsimonious explanation entails directional introgression of mitochondria from *B. fowleri* into *B. americanus*. Central clade individuals from both Pennsylvania and Tennessee were collected in the field amid *B. fowleri* individuals whose sequences appear in the South or North clades (Figs. 3 and 5). In addition, no morphologically pure (hybrid score = 4A) *B. americanus* possessed the Central clade haplotype.

What could account for the three genetically divergent mtDNA clades, given that no phenotypic differences between northern, central, and southern populations of *B. fowleri* are known? Our data motivate three hypotheses; each scenario begins with an ancestral population splitting into three populations about 2 mya, with subsequent genetic divergence, followed later by divergence between *B. terrestris* and the southern clade of *B. fowleri*.

One hypothesis is that mtDNA differentiation may not have been accompanied by morphological differentiation. *Bufo* are characterized by relatively conserved morphological features, despite ancient genetic divergences (Wallace et al., 1971; Wilson et al., 1974). If this hypothesis were correct, we may expect sequences from nuclear loci to mirror our pattern of geographically structured divergence among the three *B. fowleri* clades.

Alternatively, perhaps the three lineages of *B. fowleri* did diverge phenotypically, but secondary contact with subsequent extensive hybridization homogenized their morphological distinctions. If this were true, we would expect nuclear sequences not to exhibit geographic structure.

Lastly, perhaps *B. fowleri* populations diverged phenotypically and remain so today, despite limited secondary contact and hybridization, but subtle differences have been overlooked. This may be possible, as geographic variation has not been systematically examined, despite the long history of study of these bufonids (Brown, 1964, 1970a; Cory and Manion, 1955; Green, 1982, 1984; Jones, 1973; Meacham, 1962; Myers, 1931; Volpe, 1952, 1959). To discriminate among these three hypotheses, future work should focus on whether phenotypic differences exist and whether there is corresponding divergence in nuclear genes.

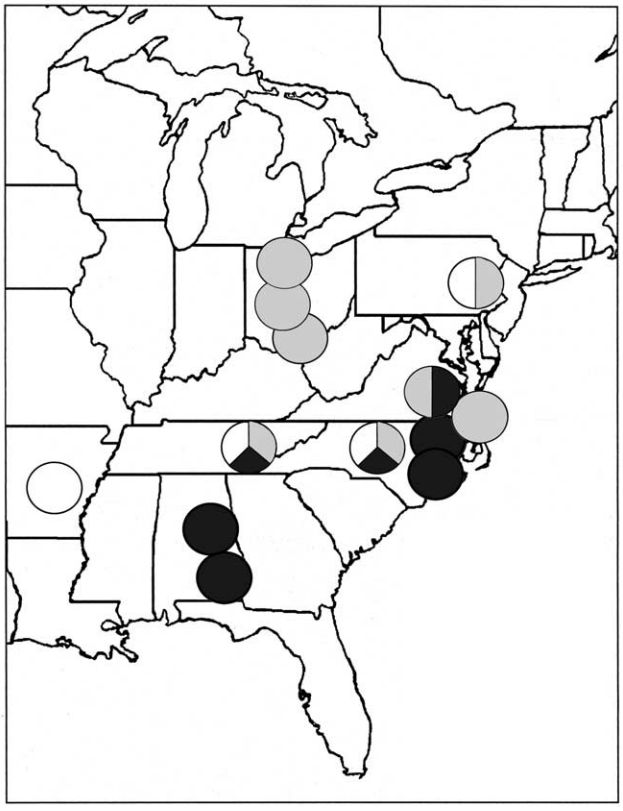

Fig. 5. Geographic locations of the mitochondrial haplotypes from three clades of *B. fowleri* (North, South, and Central) recovered in this study. Circles represent collection locations, with dark gray representing haplotypes corresponding to the southern clade, light gray the northern clade, and unfilled circles the Central clade. Some populations (Pennsylvania and Virginia) contained haplotypes belonging to two clades, represented by half circles, while the Tennessee and North Carolina populations contained haplotypes from all three clades.

4.2. Hybridization

Among lineages we recovered, mitochondria from *B. woodhousii* appear to have introgressed into *B. americanus* on three separate occasions, while mitochondria from *B. americanus* have introgressed into *B. woodhousii* twice. These cases of introgression involve individuals from known areas of range overlap. Thus, in these collections of *B. americanus* and *B. woodhousii*, hybrid individuals appear to be restricted to a somewhat narrow zone that is concordant with the area of sympatry depicted in Conant and Collins (1991). Introgression was absent outside this zone of sympatry, suggesting that these two species maintain independent evolutionary trajectories in allopatry. The absence of mitochondrial gene flow outside the hybrid zone could also indicate selection against hybrids.

Louisiana populations, although composed of individuals that were morphologically *B. americanus*, *B. fowleri*, or hybrids, did not possess mitochondrial genes of *B. fowleri*, but instead fell within the *B. americanus* or *B. woodhousii* clades. This suggests that these populations include hybrids containing nuclear genes from all three species. Haplotypes of *B. fowleri* were found no further west than Hot Springs, Arkansas, whereas *B. woodhousii* haplotypes were found east to southeast Illinois. This suggests that the mitochondria of *B. woodhousii* are introgressing further east than previously suspected (see range map, Fig. 1), whereas *B. fowleri* genes may not have introgressed as far west as is currently believed (Conant and Collins, 1991), although further sampling is necessary to ascertain this.

The picture is even more complicated for *B. fowleri* across most of the remainder of its range, with respect to the sympatric *B. americanus*. Not surprisingly, more introgression has occurred between these species, such that it cannot be localized to a single geographic area. While some individuals are clearly hybrids (Table 1), four show the morphological features of a "pure" *B. fowleri*, yet have mitochondria that appear to have come from *B. americanus*. These individuals may be descendants of an old hybridization event, followed by subsequent backcrossing with *B. fowleri* sufficient to retain the *B. fowleri* morphology. Alternatively, they may be of recent hybrid origin, as little is known about the morphological appearance of F1 and F2 hybrids between these species (however, see Jones, 1973).

The amount of hybridization among these toads has evidently been quite high historically: over 12% of the morphologically defined *B. americanus* specimens possessed haplotypes of *B. woodhousii* or *B. fowleri*, whereas 20% of the morphologically defined *B. fowleri* possessed haplotypes of *B. woodhousii* or *B. americanus*. An additional 12 individuals were scored as morphological hybrids between *B. fowleri* and *B. americanus* or *B. fowleri* and *B. woodhousii*. Because only large cranial crests can be used to safely distinguish pure *B. terrestris* (Volpe, 1959), we were unable to confidently assess the extent of hybridization with this species. The above figures are probably underestimates, for two reasons. The choice of a threshold for designating individuals as hybrids or pure-bred is necessarily subjective and our decision to score toads as hybrids if they possessed fewer than three of four traits characterizing a species certainly influences our estimates of the number of hybrid individuals. Had we used a higher threshold, we would have identified *more* of our specimens from the outset as hybrids. Therefore, our estimates are conservative. Second, because mtDNA can only inform us of the maternal pedigree, an analysis based on mtDNA sequences alone can underestimate the true amount of cryptic hybridization, especially if the hybrids closely resemble the maternal parental species.

Hybrids of *Bufo* have traditionally been identified by their intermediate appearance or call. Our results therefore call into question previous analyses of *B. americanus* group taxa that were based solely on phe-

notype, as this study provides evidence that an individual can show all the morphological traits of one species, yet possess the mtDNA haplotype of another.

4.3. Call evolution

Our explicit phylogenetic hypothesis inferred from molecular data allows us to examine patterns of phenotypic character evolution, such as those of advertisement or mating calls. The mating call of *B. americanus* has fewer pulses per second and a longer call duration than that of *B. fowleri* (Blair, 1958; Green, 1982; Jones, 1973) or *B. woodhousii* (Sullivan et al., 1996), as does the mating call of *B. terrestris* (Brown, 1970b; Cocroft and Ryan, 1995). The mating call of *B. microscaphus* has a pulse rate similar to *B. americanus* but a duration intermediate between *B. americanus* and *B. woodhousii* (Blair, 1957, 1958; Cocroft and Ryan, 1995; Sullivan et al., 1996). Thus, with our mtDNA phylogeny one would infer by parsimony that long calls evolved independently in *B. americanus* and *B. terrestris* (see Fig. 4). If *B. hemiophrys* with its intermediate-duration call is the sister taxon to *B. americanus* as suggested by Goebel (1996), then short calls may also have evolved in parallel in *B. woodhousii* and *B. fowleri*.

This parallelism suggests that selection may have acted on advertisement call variation. Call evolution in anurans may often be shaped by species interactions or sexual selection (Blair, 1958; Cocroft and Ryan, 1995). Potentially, divergent character displacement in sympatry could also account for the call differences among these taxa, although evidence for this is not conclusive (Leary, 2001). Alternatively, the parallelism may simply be a non-selective result of chance evolution.

4.4. Implications for "Bufo fowleri"

While the results presented here do not completely resolve the systematic placement or population genetic structure of *B. fowleri*, they do indicate that the traditional understanding of this taxon will need substantial revision. Clearly, *B. fowleri* can no longer be viewed as a subspecies of *B. woodhousii*, as the genetic evidence demonstrates it to be less related to *B. woodhousii* than to *B. terrestris*. For the same reason, the two taxa cannot be considered sister species, as has been alternatively proposed.

Because *B. terrestris* is nested within *B. fowleri*, avoidance of paraphyletic taxa would necessitate either subsuming *B. terrestris* into *B. fowleri* or elevating each of the three clades of *B. fowleri* to species status. Such decisions should perhaps await further sampling, once the phylogeographic structure of *B. fowleri* has been more thoroughly detailed and the amount of nuclear gene divergence and introgression among these populations is better understood.

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