GENETIC VARIATION AND POPULATION STRUCTURE IN THE ENDANGERED HOUSTON TOAD IN CONTRAST TO ITS COMMON SYMPATRIC RELATIVE, THE COASTAL PLAIN TOAD

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by

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 2 Page

LIST OF FIGURES

GENETIC VARIATION AND POPULATION STRUCTURE IN THE ENDANGERED HOUSTON TOAD IN CONTRAST TO ITS COMMON SYMPATRIC RELATIVE, THE COASTAL PLAIN TOAD

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ABSTRACT

This is the first study to assess genetic variation in the endangered Houston toad, *Bufo houstonensis* (Anura: Bufonidae). Samples from across its range were used to determine the number of populations and the levels of diversity within and among populations. D-loop (mtDNA) sequences from 160 individuals were analyzed, and variation at ten microsatellite loci was examined for 439 individuals. Genetic clustering analyses indicate nine populations across the range; five of these populations occurred in Bastrop County. The most divergent population was in Austin County. Gene flow was generally low, but was higher at distances <4 km. Overall, genetic diversity was high across the range and within populations. Recent surveys indicate population sizes are low and are decreasing; accordingly, annual monitoring of all known populations and increasing the number of toads (e.g., through headstarting programs) are proposed for immediate implementation. More general, but crucial, recommendations include preservation of all three habitat types (breeding/nursery, occupied, and dispersal), special attention towards the Austin County population, and involvement of the general public in conservation.

The coastal plain toad, *Bufo nebulifer*, is sympatric with *B. houstonensis* through all of the latter species' range. Examination of more common sympatric congeners may be

necessary to effectively manage rare or endangered species, especially in cases where widespread or frequent hybridization is known or when human activities increases the rates of hybridization. *Bufo houstonensis* and *B. nebulifer* are known to hybridize, and while recent work has been done to investigate the genetic diversity and structure within *B. houstonensis*, no comparable data yet exist for *B. nebulifer*. I investigated population genetic structure and diversity, including migration/movement rates, at both the landscape and fine scales. Much of the range was sampled and nine groups were recovered. Their relationships may be explained by a long residence in much of its present-day distribution (at least tens of thousands of years), with a history of range contraction during glaciation and re-expansion following the retreat of glaciers. *Bufo houstonensis* and *B. nebulifer* have comparable levels of genetic diversity, but *B. nebulifer* seems to migrate less frequently or less distance than its endangered congener.

In order to effectively protect endangered species, natural levels of interspecific hybridization, or admixture, must be characterized, especially in cases where anthropogenic changes to the environment may broaden contact between the species. I investigated the baseline levels of admixture in *B. houstonensis* using mitochondrial sequence data and microsatellite loci. Admixture between *B. houstonensis* and two sympatric species (*B. nebulifer* and *B. woodhousii*) was detected. Phenotype-based assessments of admixture appear to be temporally stable, but they underestimate true levels of admixture. *Bufo nebulifer* × *B. houstonensis* F1 hybrids can be fertile and backcross to *B. nebulifer*; *B. nebulifer* × *B. houstonensis* matings may result in fertile offspring more frequently than previously thought. Admixed individuals with *B. houstonensis* or *B. woodhousii* maternal lineages can backcross to *B. houstonensis*. Phenotypically aberrant individuals were not always F1 hybrids, and F1 hybrids were not always phenotypically aberrant. With continued habitat alteration and rising temperatures, both habitat isolation and offset breeding season have already partially

broken down and may deteriorate further, consequently, opportunities for hybridization events will increase. Selection against hybrids at the tadpole stage did not occur among *B*. *houstonensis* and *B*. *nebulifer* individuals. All these factors may also lead to higher levels of gamete wastage in *B*. *houstonensis*, an already critically endangered species.

Thirty-five published microsatellite loci were screened in several *Bufo* species, chiefly *B*. *houstonensis* and its two common, sympatric relatives, *B*. *nebulifer* and *B*. *woodhousii*. Twelve loci were polymorphic in the three focal species. For some loci, amplification was observed in distantly related species. Natural hybridization occurs within the genus *Bufo* and laboratory crosses often result in viable or fertile offspring. These microsatellite loci may be used to address questions of interspecific admixture as well as baseline intraspecific genetic variation.

Chapter 1

INTRODUCTION

Amphibians are in decline worldwide (Blaustein et al. 1994; Alford & Richards 1999; Blaustein & Kiesecker 2002). McCallum (2007) estimated that extinction rates for the past 500 years are more than 20 times greater than background extinction rates for amphibians. Indeed, more than 1200 amphibians are listed as endangered or critically endangered by the IUCN (Stuart et al. 2004; Wake & Vredenburg 2008; IUCN 2009). Determining why current declines are happening is the focus of much multi-disciplinary research (Semlitsch 2003).

Assessment of genetic diversity in declining species can reveal basic information about population structure, levels of diversity at the species and population levels, and connectivity or gene flow among populations; this information can help inform why some species are declining and others are not. Until recently, rare species were thought to be genetically impoverished compared to more widespread species (Gitzendanner & Soltis 2000), but with the advent of cheaper and more efficient molecular tools (Hedrick & Miller 1992; Jehle & Arntzen 2002), we are finding that genetic diversity is very high even among the rarest species. This high genetic variation may mean that, for many endangered amphibians, declines are not caused by low genetic diversity but instead are a result of other threats such as climatic change, disease, or anthropogenic habitat modifications. A growing body of recent research highlights how phylogeographic and

population genetic studies can help direct management strategies of endangered or declining amphibians (e.g., Shaffer et al. 2000; Monsen & Blouin 2004; Measey et al. 2007; Morgan et al. 2008; Goebel et al. 2009; Wang 2009).

The Houston toad, *Bufo houstonensis* (= *Anaxyrus houstonensis*, Frost et al. 2006a) is endemic to southeast-central Texas and is listed as endangered at the State and Federal levels (Gottschalk 1970; Potter et al. 1984). A long-term downward trend in *B. houstonensis* is apparent with both decreasing numbers of individuals and decreasing numbers of populations. A translocation program conducted by the Houston Zoo in the 1980s as part of the Houston Toad Recovery Plan sought to reintroduce *B. houstonensis* into new sites within the historical range (Quinn 1980; Potter et al. 1984; Quinn et al. 1984; Quinn et al. 1987) but no new populations had been established as of 1991 (Dodd & Seigel 1991). Current work is underway to establish a new supplementation program at the Houston Zoo designed to increase juvenile survival at natal ponds through headstarting (Forstner et al. 2007). Understanding the population genetic structure will improve the efficacy of programs such as this by providing basic information about divergences, migration or dispersal patterns, and levels of genetic variation among populations.

Despite the known rangewide long-term population decline and despite it being the first amphibian listed as Federally endangered, no attempts have been made to assess genetic variation at any scale within *B. houstonensis*. Few genetic studies have been performed, all of which used protein electrophoresis (Thomas & Dessauer 1982; Hillis et al. 1984; Hillis & Price 1993). Managers need to know how genetic variation is

partitioned to effectively administer the few remaining populations of *B*. *houstonensis*. While it may be possible to stabilize wild populations, long-term trends indicate that captive breeding colonies, like those for *Peltophryne lemur* (Beauclerc et al. 2010) or *Bufo baxteri* (Browne et al. 2006), may be a necessary component in the conservation of *B*. *houstonensis*. In Chapter 2, the following questions are addressed using sequence data and microsatellite loci: (1) where does *B*. *houstonensis* fit in the *B*. *americanus* species group? (2) what is a population in *B*. *houstonensis* and how many exist? (3) what are the levels of genetic diversity within and among populations? (4) how differentiated are populations? and (5) what are the patterns of migration at the landscape- and fine-scale levels?

For endangered or rare taxa that occur in sympatry with common and/or abundant congeners, hybridization can be a threat to the survival of the species Accordingly, in addition to understanding the biology of a rare species, we must also investigate its sympatric congeners. This is especially important in taxa with widespread or frequent hybridization or when human activities may be increasing the rates of hybridization (e.g., introduction of nonnatives or habitat alteration). Hybridization among toad species in the family Bufonidae is well-known and widespread (Blair 1959, 1963; Brown 1971; Blair 1972a; Gergus et al. 1999; Vogel & Johnson 2008); hence, for the endangered *B*. *houstonensis*, examination of its abundant sympatric relatives is essential to its recovery effort.

The coastal plain toad, *Bufo nebulifer* (= *Incilius nebulifer*, Mulcahy & Mendelson 2000; Frost et al. 2006a; Frost et al. 2006b; Frost et al. 2009), is a common

and abundant toad throughout its range (from Veracruz, Mexico into northern Texas and from the Big Bend area in Texas east to Louisiana; Hammerson & Canseco-Márquez 2004; IUCN 2009) occurring throughout the entire range of *B*. *houstonensis*, sometimes chorusing at the same pond at the same time (Brown 1971; Hillis et al. 1984; Price 1990; Forstner 2002). In Chapter 3, the population genetic structure within *B*. *nebulifer* is investigated for the first time. Besides expanding knowledge of this common and successful species, understanding its genetic diversity and structure may provide insight into why *B*. *houstonensis* is rare and how that endangered taxon may be more effectively managed. To truly appreciate the diversity and structure within a rare species, it should be placed in the context of its relatives (Karron 1987; Gitzendanner & Soltis 2000), particularly those in sympatry. Coupling genetic diversity and structure of both species with what has already been determined about their life histories may reveal why some species are common and others are rare. In Chapter 3, the following questions are addressed: (1) what is a population in *B*. *nebulifer* and how many exist? (2) what are the levels of genetic diversity within and among populations? (3) how differentiated are populations? and (4) what are the patterns of gene flow at the landscape and fine scales?

Detecting hybridization, or admixture, between taxa is key for conservation management of endangered species. Admixture threatens endangered or rare taxa via gamete wastage, population-wide lowered fitness due to the presence of less fit hybrids, and extinction through introgression or through competition with heterotic hybrids. Hybridization is a natural occurrence for many taxa. However, admixture resulting from human-caused changes in the environment has also been reported (Levin et al. 1996;

Allendorf et al. 2001; Fitzpatrick & Shaffer 2007). To understand the implications of human-induced hybridization and to manage endangered taxa, levels of natural admixture must be characterized.

Admixture among toad species in the family Bufonidae is known to occur naturally (Blair 1972a), but a few recent studies have implicated anthropogenic causes in some hybridization events (Gergus et al. 1999; Dixon 2000; Vogel & Johnson 2008). The endangered Texas endemic *B. houstonensis* naturally hybridizes with *B. nebulifer* and with *Bufo woodhousii* (= *Anaxyrus woodhousii*, Frost et al. 2006a) (Brown 1971; Hillis et al. 1984), and laboratory crosses with other bufonid species result in viable or fertile offspring (Blair 1959, 1963, 1972a). Levels of admixture are still unknown (i.e. are they F_1 hybrids, F_2 hybrids, and/or backcrosses?), and characterizing baseline levels of natural hybridization is necessary to assess the full impact of current and future anthropogenic habitat modification and consequent changes in interspecific admixture. These baseline levels are investigated in Chapter 4 using mitochondrial sequence data and nuclear microsatellite loci. In addition to determining how much admixture exists in *B. houstonensis*, the types of hybrid classes, the temporal and geographic limits of admixture, and selection against hybrids at different life-history stages were also examined.

The ~250 species in the toad genus *Bufo* sensu lato (for recent taxonomic changes within the genus see Frost et al. 2006a; Frost et al. 2006b; Frost et al. 2009) are found nearly world-wide and occupy a broad variety of habitats (Blair 1972b). According to the IUCN Red List of Threatened Species, 31 are Endangered and 10 are Critically

Endangered (IUCN 2009). Thirty-five microsatellite loci from the literature were tested in multiple bufonid species: *Bufo bufo*, *Bufo boreas*, *Bufo cognatus*, and *Bufo marinus*. In addition to the three focal species, *B. houstonensis*, *B. nebulifer*, and *B. woodhousii*, 11 other species in the genus were also screened to evaluate the utility of these markers in New Worlds bufonids.

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Chapter 2

MICROSATELLITE AND MTDNA ANALYSES REVEAL HIGH GENETIC DIVERSITY AND MULTIPLE POPULATIONS OF THE HOUSTON TOAD (*BUFO HOUSTONENSIS*)

Abstract This is the first study to assess genetic variation in the endangered Houston toad, *Bufo houstonensis* (Anura: Bufonidae). Samples from across its range were used to determine the number of populations present and the levels of diversity within and among populations. D-loop (mtDNA) sequences from 160 individuals were analysed, and variation at ten microsatellite loci was examined for 439 individuals. Genetic clustering analyses indicate nine populations across the range; five of these populations occurred in Bastrop County. The most divergent population was in Austin County (F_{ST} = 0.196-0.400). Gene flow was generally low, but was higher at distances <4 km. Toads at one site are probably descendants of toads that were translocated from 100+ km NW in the 1980s. Overall, genetic diversity was high across the range and within populations. Recent surveys indicate that population sizes are low and are decreasing; accordingly, annual monitoring of all known populations and increasing the number of toads (e.g., through headstarting programs) are proposed for immediate implementation. More general, but crucial, recommendations include the preservation of all three habitat types (breeding/nursery, occupied, and dispersal), special attention towards the Austin County population, and involvement of the general public in conservation of *B. houstonensis*.

Introduction

Amphibians are in decline worldwide (Blaustein et al. 1994; Alford & Richards 1999; Blaustein & Kiesecker 2002). McCallum (2007) estimated that extinction rates for the past 500 years are more than 20 times greater than background extinction rates for amphibians. Indeed, more than 1200 amphibians are listed as endangered or critically endangered by the IUCN (Stuart et al. 2004; Wake & Vredenburg 2008; IUCN 2009). Determining why current declines are happening is the focus of much multi-disciplinary research. Assessment of genetic diversity in declining species can reveal basic information about population structure, levels of diversity at the species and population levels, and connectivity or gene flow among populations; this information can help inform why some species are declining and others are not. Until recently, rarer species were thought to be genetically impoverished compared to more widespread species (Gitzendanner & Soltis 2000), but with the advent of cheaper and more efficient molecular tools (Hedrick & Miller 1992; Jehle & Arntzen 2002), we are finding that genetic diversity is very high even among the rarest species. Kraaijeveld-Smit et al. (2005) found high genetic variation in *Alytes muletensis*, the Mallorcan midwife toad, using microsatellites. And the critically endangered Puerto Rican crested toad, *Peltophryne lemur*, has within-population genetic diversity similar to or greater than other amphibians (Beauclerc et al. 2010). This high genetic variation may mean that, for many endangered amphibians, declines are not caused by low genetic diversity but instead are a result of other threats such as climatic change, disease, or anthropogenic habitat modifications. Recent research highlights how phylogeographic and population

genetic studies can help direct management strategies of endangered or declining amphibians (e.g., Shaffer et al. 2000; Monsen & Blouin 2004; Measey et al. 2007; Morgan et al. 2008; Goebel et al. 2009; Wang 2009). The success of recommendations from these types of studies is as yet unknown for most taxa; but, for *P*. *lemur*, recent work shows that one captive breeding colony has diverged very little from the wild population indicating that those toads are still suitable for reintroduction efforts (Beauclerc et al. 2010).

The Houston toad, *Bufo houstonensis* (= *Anaxyrus houstonensis*, Frost et al. 2006) is endemic to southeast-central Texas (Figs. 1a, 1b) and is listed as endangered at the State and Federal levels (Gottschalk 1970; Potter et al. 1984; Campbell 2003). Many aspects of its biology have been studied: reproductive ecology (Hillis et al. 1984), growth (Quinn & Mengden 1984; Greuter & Forstner 2003), abiotic pond characteristics (Forstner & Ahlbrandt 2003), juvenile behavior (Thomas & Allen 1997), and hybridization (Blair 1963; Brown 1971; Hillis et al. 1984). A long-term downward trend in *B*. *houstonensis* is apparent with both decreasing numbers of individuals and decreasing numbers of populations (Table 1). *Bufo houstonensis* has been extirpated from one-quarter of its known historical range for over 30 years (Fig. 1a, Table 1). A translocation program conducted by the Houston Zoo in the 1980s as part of the Houston Toad Recovery Plan sought to reintroduce *B*. *houstonensis* into new sites within the historical range (Quinn 1980; Quinn et al. 1984; Potter et al. 1984; Quinn et al. 1987) but no new populations had been established as of 1991 (Dodd & Seigel 1991). Current work is underway to establish a new supplementation program at the Houston Zoo designed to

increase juvenile survivorship through headstarting (Forstner et al. 2007a). Understanding the population genetic structure will improve the efficacy of programs such as this by providing basic information about divergences, migration or dispersal patterns, and levels of genetic variation among populations; this basic information also applies to other types of conservation techniques like habitat conservation or restoration and tracking the spread of pathogens.

Despite the known rangewide long-term population decline and despite it being the first amphibian listed as Federally endangered, no attempts have been made to assess genetic variation at any scale within *B*. *houstonensis*. Few genetic studies have been performed, all of which used protein electrophoresis: Hillis & Price (1993) found patterns of reticulate hybridization among populations of *B*. *houstonensis* and no evidence of genetic divergence among populations; Thomas & Dessauer (1982) compared *B*. *houstonensis* with *Bufo americanus charlesmithi*, but only four *B*. *houstonensis* samples were taken from one collection site; and allozymes were also used to measure hybridization among congeners (Hillis et al. 1984). Managers need to know how genetic variation is partitioned to effectively administer the few remaining populations of *B*. *houstonensis*. While it may be possible to stabilize wild populations, long-term trends indicate that captive breeding colonies, like those for *P*. *lemur* (Beauclerc et al. 2010) or *Bufo baxteri* (Browne et al. 2006), may be a necessary component in the conservation of *B*. *houstonensis*. In my study, the following questions are addressed using mitochondrial sequence data and nuclear microsatellite loci: (1) where does *B*. *houstonensis* fit in the *B*. *americanus* species group? (2) what is a population in *B*. *houstonensis* and how many

exist? (3) what are the levels of genetic diversity within and among populations? (4) how differentiated are populations? and (5) what are the patterns of migration at the landscape and fine scale levels?

Materials and methods

Sampling

Individuals were sampled across southeast-central Texas, the historical range of *B. houstonensis*, from 2000 to 2008 (Appendix A). In two areas in Bastrop County, Griffith League Ranch (GLR) and Bastrop State Park (BSP), multi-year trapping studies were conducted during which tissue was collected (Forstner & Swannack 2004; Jones 2006). Forty-eight ponds and/or sites were sampled within Bastrop County which houses the largest numbers of remaining *B. houstonensis* (Fig. 1, Table 2, Hillis et al. 1984; Potter et al. 1984). Considerably fewer ponds and/or sites were sampled in other counties: three in Austin, one in Colorado, three in Lee, one in Leon, and one in Milam (Table 2). No individuals were observed in other counties within the range of *B. houstonensis* from 2000 to 2008.

Tissue sampling was non-consumptive where possible. Toe clip or blood tissue samples were collected from live adult toads (muscle or skin was taken from vouchered animals), and some eggs and tadpole tails were sampled. Blood samples were stored at –80 °C in a blood storage buffer modified from Longmire et al. (1988): 100 mM TRIS, 100 mM EDTA disodium dihydrate, 1 % w/v sodium dodecyl sulfate, pH = 8.0. Toe clips, muscle, skin, eggs, and tadpoles were stored in 96 % ethanol at –80°C. Tissues

were deposited in the Michael R. J. Forstner Frozen Tissue catalog at Texas State University—San Marcos. Vouchered specimens were deposited at the Texas Cooperative Wildlife Collection.

Bufo houstonensis were sampled under Department of the Interior, U.S. Fish and Wildlife Service, Federal Fish and Wildlife Permit Numbers TE039544-1, TE039544-2, TE004472-0, and TE004472-1 and Texas Parks and Wildlife Scientific Permit Numbers SPR-0102-191 and SPR-0290-022 and under Institutional Animal Care and Use Committee approvals 5Qrs45_02, HGVMAD_02, 04-0485904A30, 0713_0428_07, and 0810_0208_11.

DNA extraction

DNA was isolated from tissue (1-2 mm^3 toe clip, muscle, skin, tadpole tail; 10-50 μl blood in storage buffer; egg excluding gelatinous layer) using a Wizard® SV 96 Genomic DNA Purification System (Promega) on a Biomek® 3000 Laboratory Automation Workstation (Beckman Coulter), or using a DNeasy® Tissue Kit (QIAGEN Inc.), following manufacturer's instructions for both, or using a standard phenol-chloroform method (Sambrook et al. 1989). DNA extractions were assessed by agarose gel electrophoresis and were visualized following ethidium bromide staining under UV light.

Sequences

A 533 base pair (bp) fragment of the control region (D-loop) of the mitochondrial

genome (mtDNA) was sequenced. Amplification was performed using the primers BHDL1 (5'-TGCATATCATCACCAATCC-3') and BUFOR1 (5'-CTGAGGCCGCTTTAAGGTACGATAG-3') in reactions with 4 mmol $MgCl_2$, 0.1 mM dNTPs, 0.01 μM each primer, 2.5 units *Taq* polymerase, and pH = 8.5. PCR was performed with an initial denaturing period of 95°C for 5 min then 35 cycles, each consisting of denaturing at 95°C for 30 sec, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, and a final extension period of 72 °C for 5 min. Positive and negative controls were used. PCR products were purified with an AMPure® PCR Purification System (Agencourt Bioscience Corporation), and then cycle sequenced with the above primers, using a CEQ™ DTCS Quick Start Kit (Beckman Coulter) following manufacturer's instructions. Thermal cycling was 30 cycles of 96°C for 20 sec, 50 °C for 20 sec, and 60 °C for 4 min. Products were cleaned by ethanol precipitation (following Beckman Coulter manufacturer's instructions) and analyzed on a CEQ™ 8800 Genetic Analysis System (Beckman Coulter). Resultant sequences were edited and aligned in SEQUENCHER™ Version 4.5 (Gene Codes Corp.).

Microsatellites

Amplifications of microsatellite loci were performed using WellRED fluorescently labeled forward primers (see Table 3) in 10 μl reactions with 4 mmol $MgCl_2$, 0.1 mM dNTPs, 0.01 μM each primer, 2.5 units *Taq* polymerase, and pH = 8.5. PCR was performed with an initial denaturing period of 95 °C for 5 min then 35 cycles, each consisting of denaturing at 95 °C for 30 sec, annealing for 1 min (see Table 3 for

annealing TºC for each locus), and extension at 72 °C for 1 min, and a final extension period of 72 °C for 5 min (except locus BBR34-2 for which no initial 5 min denaturing period was used). Amplification products were electrophoresed, singly or pooled (see Table 3), on a CEQ™ 8800 Genetic Analysis System (Beckman Coulter) following manufacturer's instructions. Allele sizes were determined with CEQ™ 8800 FRAGMENT ANALYSIS software (Beckman Coulter) by eye. At least two PCR attempts were made, for each individual per locus, before scoring the locus as not amplifiable. See also Chapter 5.

Phylogenetic analyses

To assess the phylogenetic placement of *B*. *houstonensis*, maximum parsimony (MP), maximum likelihood (ML, Felsenstein 1981), and Bayesian analyses using mtDNA data were performed in which the following taxa were included (Table 4): *Bufo americanus*, *Bufo cognatus*, *Bufo fowleri*, *B*. *houstonensis*, and *Bufo woodhousii*. *Bufo cognatus* was used as an outgroup. Maximum parsimony topologies were generated using equal character weighting, Fitch parsimony, ACCTRAN optimization, heuristic search, random stepwise addition sequence (10,000 replicates), tree bisection-reconnection (TBR) branch swapping, and MulTrees in PAUP* version 4.0b10 (Swofford 2002). Multiple equally parsimonious trees were summarized using strict consensus. Model parameters for maximum likelihood, which were estimated by hLRT and AIC using MODELTEST version 3.7 (Posada & Crandall 1998), were used as input in a ML heuristic search in PAUP* version 4.0b10 (Swofford 2002). Bootstrap values (Felsenstein 1985) were estimated from 100 replicates in a heuristic search with random stepwise addition sequence (ten

replicates) and TBR branch swapping in PAUP* version 4.0b10 (Swofford 2002) for MP and ML analyses. Parameters of a best-fit nucleotide model of evolution for Bayesian analysis were determined by hLRT and AIC in MRMODELTEST version 2.0 (Nylander 2004), and MRBAYES version 3.1.2 (Ronquist & Huelsenbeck 2003) was implemented for ten million generations, saving every thousandth tree, and with a burn-in of 2,500 trees.

To assess intraspecific relationships, a statistical parsimony network (Templeton et al. 1992) of mtDNA haplotypes in *B. houstonensis* was constructed using TCS version 1.21 (Clement et al. 2000).

Genetic clustering analyses

GENELAND analyses

GENELAND version 3.1.4 (Guillot et al. 2005a; Guillot et al. 2005b; Guillot 2008; Guillot et al. 2008) was used to infer the number of clusters (*K*), or populations, in the dataset and to assign individuals to a cluster. To determine the number of clusters, ten independent runs were performed, wherein ploidy was two, loci were codominant, maximum rate of Poisson process was equal to the number of individuals in the dataset, uncertainty on coordinates was 0.0015, number of populations (*K*) was allowed to vary from 1 to 10, maximum number of nuclei was three times the number of individuals in the dataset, the allele frequency model was uncorrelated (= Dirichlet), 1,000 stored iterations (1,100,000 iterations, 1,000 thinning, 100 burn-in) were used, the null allele model was not used, and the spatial model was used.

Guillot et al. (2005a) suggested setting the maximum rate of Poisson process (rate.max) equal to the number of individuals and the maximum number of nuclei (nb.nuclei.max) equal to three times the number of individuals. The uncertainty on coordinates (delta.coord) was set to 0.0015, because this is approximately equivalent to 150 m which was the largest possible error when data were collected in the field. The uncorrelated allele frequency model was used because it has been shown to outperform the alternative model, especially for systems with weak differentiation among clusters (Guillot et al. 2005a); in addition, using the correlated allele frequency model resulted in positive average logarithm posterior densities (data not shown).

To assign individuals to a cluster, 100 independent runs were performed using the above parameters, except number of populations was set to the modal value determined in the initial runs. The 100 runs were ranked by their average logarithm of posterior density, and the posterior probabilities from the best ten runs (i.e., had the highest average logarithm posterior density) were used to assess population membership (after post-processing with 100×100 pixels in the spatial domain. Within a run, individuals were unambiguously assigned to a cluster membership if the posterior probabilities were ≥ 0.8; individuals with posterior probabilities <0.8 were assigned to membership in multiple clusters. The posterior probabilities from the best ten runs were compared visually; the modal memberships were used as assignments. When no modal membership existed, individuals were assigned to multiple clusters. A comparison of genetic clustering analyses is presented in Table 5. The analysis of the dataset that included all individuals ($n = 439$) was designated analysis A. A similar analysis was also run (analysis

B) where the spatial model was not used.

Some loci have many missing data, even after multiple attempts at PCR; to assess whether the results were biased by missing data, a subset of individuals (those with no missing data) was analysed as above.

A large majority of the samples (97.3 %) were collected in Bastrop and Lee counties (n = 416 and n = 11, see Table 2). To determine if oversampling in Bastrop and Lee counties was biasing the results, ten other analyses were performed. Subsets were constructed, in which individuals from all other counties (Austin, Colorado, Leon, and Milam) were always included (n = 12) and 20 randomly selected individuals from Bastrop and Lee counties were included. Analyses were performed using these ten subsets, to determine K and then to assign individuals to clusters, as described above.

Because most *B. houstonensis* currently are found in Bastrop County (Hillis et al. 1984; Potter et al. 1984), and many at least used to occur in Lee County (Michael R. J. Forstner, personal communication), an analysis was performed on individuals from only Bastrop and Lee counties (n = 427), as described above (analysis C). A similar analysis was also run (analysis D) where the spatial model was not used.

To determine if GENELAND was detecting only the uppermost hierarchical level of genetic structure, two second-order analyses were performed (analysis E); similar analyses were also run (analysis F) where the spatial model was not used. The first included individuals assigned to one cluster (cluster N as determined by GENELAND, see results, n = 195); these individuals were from GLR, the Musgrave property, and Highway 290 at Sandy Creek, Bastrop County, Texas (sites BAN01p, BAN02p, BAN03s,

BAN04p, BAN05p, BAN06p, BAN07p, BAN08p, BAN10p, BAN12t, BAN13t, BAN14t, BAN15t, BAN16t, BAN17t, BAN18t, BAN19t, BAN20t, BAN21t, BAN23t, BAN24t, BAN25t, BAN26t, and BAN28p). The second analysis included individuals assigned to another cluster (cluster S, see results, $n = 154$); these individuals were from BSP, Bluebonnet Headquarters, and the Jim Small property, Bastrop County, Texas (sites BAS01p, BAS02p, BAS03s, BAS04p, BAS05s, BAS07p, BAS08p, BAS09p, BAS10t, BAS11t, BAS12t, BAS13t, BAS14p, BAS15p, BAS16p, BAS17p, and BAS18p). For each analysis, individuals with partial memberships in multiple clusters and individuals assigned membership in a different cluster (as determined by analysis A in GENELAND) were excluded from the dataset.

A final analysis, using mtDNA sequence data ($n = 107$), was also performed, wherein ploidy was one but all other parameters were the same as above. All individuals were genotyped ($n = 439$) but only 107 were sequenced.

STRUCTURE analyses

STRUCTURE version 2.1 (Pritchard et al. 2000) was used to infer the number of clusters (K), or populations, in the dataset and to assign individuals to a cluster. To determine the number of clusters, ten iterations at each value of K were run, from $K = 1$ to $K = 5$ ($K = 1$ to $K = 10$ were used for the dataset with all individuals, $n = 439$, and for three of the subsets used in determining if oversampling was biasing the results, see below), wherein the admixture ancestry model was used, the correlated allele frequency model was used, burn-in was 100,000, number of MCMC reps after burn-in was 1,000,000, and all other

parameters were set to default values. Falush et al. (2003) suggest using the admixture model and correlated allele frequencies model in situations where there is weak or subtle population structure, which is the most likely scenario in *B*. *houstonensis* (Hillis & Price 1993). The ad hoc measures of Evanno (2005) were used to infer the most appropriate value of *K*. Individual population assignments were made from the *Q* values (the estimated membership coefficient for each individual for each cluster) resulting from the iteration with the highest average log-likelihood for the chosen *K*. Individuals were unambiguously assigned to a cluster membership if the *Q* values were ≥0.8, and individuals with *Q* values <0.8 were assigned to membership in multiple clusters. The analysis of the dataset that included all individuals ($n = 439$) was analysis G.

Some loci have many missing data, even after multiple attempts at PCR; to assess whether the results were biased by missing data, a subset of individuals (those with no missing data) was analysed as above.

A large majority of the samples (97.3 %) were collected in Bastrop and Lee counties ($n = 416$ and $n = 11$, see Table 2). To determine if oversampling in Bastrop and Lee counties was biasing the results, ten other analyses were performed. Subsets were constructed, in which individuals from all other counties (Austin, Colorado, Leon, and Milam) were always included ($n = 12$) and 20 randomly selected individuals from Bastrop and Lee counties were included. Analyses were performed using these ten subsets, to determine *K* and then to assign individuals to clusters, as described above.

Because most *B*. *houstonensis* currently are found in Bastrop County (Hillis et al. 1984; Potter et al. 1984), and many at least used to occur in Lee County (Michael R. J.

Forstner, personal communication), an analysis was performed on individuals from only Bastrop and Lee counties (n = 427), as described above (analysis H).

To determine if STRUCTURE was detecting only the uppermost hierarchical level of genetic structure, two second-order analyses (analysis I) were performed (Evanno et al. 2005). The first included individuals assigned to one cluster (cluster N as determined by STRUCTURE, see results, n = 163); these individuals were from GLR, the Musgrave property, and Sandy Creek, Bastrop County, Texas (sites BAN01p, BAN02p, BAN04p, BAN05p, BAN06p, BAN07p, BAN08p, BAN10p, BAN12t, BAN13t, BAN15t, BAN16t, BAN17t, BAN18t, BAN19t, BAN20t, BAN22t, BAN23t, BAN24t, BAN25t, BAN26t, BAN28p, and BAN29s). The second analysis included individuals assigned to another cluster (cluster S, see results, n = 135); these individuals were from BSP, Bluebonnet Headquarters, and the Jim Small property, Bastrop County, Texas (BAS01p, BAS02p, BAS03s, BAS04p, BAS05s, BAS07p, BAS08p, BAS09p, BAS10t, BAS11t, BAS12t, BAS14p, BAs15p, BAS16p, BAS17p, and BAS18p). For each analysis, individuals with partial memberships in multiple clusters and individuals assigned membership in a different cluster (as determined by STRUCTURE) were excluded from the dataset.

Genetic diversity analyses

Allele frequencies, number of private alleles (A_p), and allelic richness (R) were estimated using FSTAT version 2.9.3 (Goudet 2001). For allelic richness, FSTAT uses a rarefaction method to adjust for differences in sample sizes (El Mousadik & Petit 1996). Exact tests for Hardy-Weinberg equilibrium (HWE) were performed with 1,000,000 Markov chain

steps and 100,000 dememorisation steps in ARLEQUIN version 3.11 (Excoffier et al. 2005). Tests for linkage disequilibrium (LDE) among loci, within or among samples, were performed in FSTAT version 2.9.3 with 1800 or 8100 permutations (see results). Significance, of HWE and of LDE, was determined after sequential Bonferroni correction with $\alpha = 0.05$ (Rice 1989).

Differences in allele frequencies among groups of sites (identified via multiple methods: genetic clustering analyses, other genetic diversity analyses, and migration rates analyses) were assessed by computing pairwise F_{ST} in ARLEQUIN version 3.11 (Excoffier et al. 2005) with 10,000 permutations and a significance value of 0.05. Five sets were analysed:

1) clusters N and S identified by STRUCTURE ($n_N = 203$, $n_S = 184$)
2) clusters I, N, S, and U identified by GENELAND ($n_I = 4$, $n_N = 214$, $n_S = 173$, $n_U = 4$)
3) groups BAPp, BAS06p, COLs, I, LEOp, N, S_1, S_2, and U detected via multiple methods ($n_{BAPp} = 39$, $n_{BAS06p} = 17$, $n_{COLs} = 3$, $n_{LEOp} = 1$, $n_I = 4$, $n_N = 196$, $n_{S1} = 71$, $n_{S2} = 75$, $n_U = 4$)
4) Bastrop County and all others ($n_{Bastrop} = 416$, $n_{other} = 23$)
5) Austin County and all others ($n_{Austin} = 4$, $n_{other} = 435$).

For sets 1 through 3, individuals assigned to multiple clusters were excluded.

Using the microsatellite dataset, I tested for isolation-by-distance among individuals with a Mantel test (Mantel 1967) in ALLELES IN SPACE version 1.0 (AIS, Miller 2005). Six analyses were performed, with 10,000 permutations each:

1) all individuals (n = 439)
2) all individuals but with log transformed geographic distances
3) only individuals from Bastrop and Lee counties (n = 427)
4) only individuals from Bastrop and Lee counties but with log transformed geographic distances
5) only individuals from Bastrop County (n = 416)
6) only individuals from Bastrop County but with log transformed geographic distances.

Four analyses were performed using the mtDNA sequence dataset: all individuals (n = 107), all individuals but with log transformed geographic distances, only individuals from Bastrop County (n = 95), and only individuals from Bastrop County but with log transformed geographic distances.

Migration rates

Migration rates were estimated using a Bayesian, assignment test-based method, as implemented in BAYESASS version 1.3 (Wilson & Rannala 2003). BAYESASS requires <20 populations; consequently, not all sites as described in Table 2 could have been used, and groups of sites were constructed based on geographic locality and results from GENELAND analyses (Table 6). Initial analyses were performed first to determine the appropriate run length (where convergence of log-likelihood values had been reached) and then to determine the appropriate delta values for allele frequencies (P), migration rates (m), and inbreeding coefficients (F) (40-60 % change in parameter values) (Wilson

& Rannala 2003). Once these values were established, ten runs were performed, each with a different starting seed (60, 12, 55, 88, 33, 59, 29, 37, 71, 99), but all with the following input values: iterations = 3,000,000, burn-in = 1,000,000, sampling frequency = 2000, P = 0.775, m = 0.15, and F = 0.775. Distributions of log-likelihood values were compared across runs; the run with the narrowest distribution was used to assess migration rates. Migration rates from all ten runs were compared to see if they converged on a similar solution.

Another analysis was performed using only sites from the western part of GLR in Bastrop County (= group BANwest; Table 7). Initial analyses were performed first to determine the appropriate run length (where convergence of log-likelihood values had been reached) and then to determine the appropriate delta values for allele frequencies (P), migration rates (m), and inbreeding coefficients (F) (40-60 % change in parameter values; the closest this change was for P and F was 78 %) (Wilson & Rannala 2003). Once these values were established, ten runs were performed, each with a different starting seed (10, 22, 99, 281, 394, 493, 588, 678, 820, 993), but all with the following input values: iterations = 3,000,000, burn-in = 1,000,000, sampling frequency = 2000, P = 0.875, m = 0.15, and F = 0.875. Distributions of log-likelihood values were compared across runs; the run with the narrowest distribution was used to assess migration rates. Migration rates from all ten runs were compared to see if they converged on a similar solution.

For both analyses, individuals were categorized as 'resident' if assigned ≥800 times to its own group at time 0, 'immigrant' if assigned ≥800 times to another group at

time 1, 'progeny of immigrant' if assigned ≥800 times to another group at time 2, or 'non-resident' if not assigned to any one group or time ≥800 times. Additionally, if all individuals in a group were assigned to another group at time 0, then they were categorized as resident and those groups were determined to be indistinct (i.e., they should not have been analysed as separate groups).

The proportion of males that were resident was compared to the proportion of females that were resident (proportion of juveniles was also compared to that of adults). The test statistic was calculated as: $Z = \frac{\hat{p}_1 - \hat{p}_2}{SE_{H_o}(\hat{p}_1 - \hat{p}_2)}$, where $\hat{p}_1$ = proportion of one group that were resident, $\hat{p}_2$ =proportion of other group that were resident, $SE_{H_o}(\hat{p}_1 - \hat{p}_2) = \sqrt{\hat{p}(1-\hat{p})(1/n_1 + 1/n_2)}$, n_1 = total number of one group, and n_2 = total number of other group. The confidence interval (CI) for p_1-p_2 was calculated as: $\hat{p}_1 - \hat{p}_2 \mp z_{1-\alpha/2} \bullet \sqrt{\hat{p}_1(1-\hat{p}_1)/n_1 + \hat{p}_2(1-\hat{p}_2)/n_2}$, where $\hat{p} = \frac{x_1 + x_2}{n_1 + n_2}$, x_1 = number of one group that were resident, and x_2 = number of other group that were resident.

AMOVA analyses

The population genetic structure was examined using a nested hierarchical analysis of molecular variance (AMOVA) for eight strategies using microsatellite data:

1) among groups identified by STRUCTURE (analysis G; clusters N and S)
2) among groups identified by GENELAND (analysis A; clusters I, N, S, and U)
3) among six groups detected via multiple methods (genetic clustering analyses, genetic diversity analyses, and migration rates analyses; groups BAPp, BAS06p,

I, N, S, and U)

4) among groups identified across analyses in GENELAND (analyses A, C, and E; clusters I, N, S_1, S_2, and U)
5) among nine groups detected via multiple methods (genetic clustering analyses, genetic diversity analyses, and migration rates analyses; groups BAPp, BAS06p, COLs, I, LEOp, N, S_1, S_2, and U)
6) among two geographic groups (sites in Bastrop and Lee counties vs. sites in all other counties)
7) two geographic groups (sites in Austin County vs. sites in all other counties)
8) among years using sites where sample sizes were sufficiently large. For this analysis, the sites were BAN02p (*n* = 108; 2000-2006), BAN08p (*n* = 13; 2001, 2004, 2005, 2007), BAPp (*n* = 39; 2003, 2005-2007), BAS01p (*n* = 17; 2006-2007), BAS06p (*n* = 17; 2003, 2005, 2007), and BAS17p, (*n* = 19; 2006-2007) (see also Table 8).

Two AMOVAs were performed using mtDNA data: 1) among sites, and 2) among some groups in Bastrop County detected via multiple methods (genetic clustering analyses, genetic diversity analyses, and migration rates analyses; groups BAPp, N, S_1, and S_2). For microsatellite AMOVAs 1 through 4, individuals with partial memberships in multiple clusters and individuals assigned membership in a different cluster were excluded from the dataset. AMOVAs were performed in ARLEQUIN version 3.11 (Excoffier et al. 2005) and significance was tested using 10,000 permutations.

Results

Sampling

Four hundred thirty-nine *B. houstonensis* in six counties from 2000-2008 were sampled for this study (Fig. 1, Table 2, Appendix A). Males were encountered more frequently (363, 82.7 %) than females (29, 6.6 %). Twenty-six juveniles and 12 tadpoles were sampled. Of the remaining nine, two were recorded as 'female?', four did not have sex recorded, and three were individuals for which the sex could not be determined. Four individuals were sampled at three sites in Austin County, 416 at 48 sites in Bastrop, three at one site in Colorado, 11 at three sites in Lee, one at one site in Leon, and four at one site in Milam. Within Bastrop County, 206 individuals were sampled in subgroup north, 171 in subgroup south, and 39 in GLR p12. Two individuals were sampled in 2000, 34 in 2001, 78 in 2002, 28 in 2003, 26 in 2004, 64 in 2005, 73 in 2006, 130 in 2007, and four in 2008 (Table 8). Two hundred sixteen samples were toe clips, 206 blood, 12 tadpole tail, three muscle, and 2 skin. Two vouchers were deposited at the Texas Cooperative Wildlife Collection (TCWC84556, TCWC87316).

Phylogenetic analyses

The 538 bp D-loop alignment of 194 individuals (160 *B. houstonensis*) resulted in 26 unique haplotypes (GenBank Accession Nos. HM021093–HM021118). Four hundred nine characters were constant and 105 were parsimony-informative. The model of evolution that best fitted the data was HKY+G, as determined by MODELTEST and by MRMODELTEST. The Bayesian phylogram is shown in Fig. 2; two haplotypes in three

individuals of *B*. *cognatus* were used as the outgroup. MP, ML, and Bayesian analyses resulted in similar topologies; Table 9 shows the support values for clades found by all analyses. Plotting uncorrected pairwise distance (after excluding uninformative characters) against absolute number of differences reveals saturation only in comparisons between *B*. *cognatus* and *B*. *fowleri* (Fig. 3a). Saturation is evident in transitions, but again, only in comparisons involving *B*. *cognatus* (Fig. 3b). Relationships among species in the *americanus* complex (*B*. *americanus*, *B*. *fowleri*, *B*. *houstonensis*, and *B*. *woodhousii*) were unresolved. Two species were monophyletic: *B*. *fowleri* and *B*. *woodhousii*. Fourteen *B*. *houstonensis* haplotypes were found in clades Ib, Ic, Id, IIIa (22 of 27 individuals), and IIIb (3 of 5 individuals in wooC). Five *B*. *americanus* haplotypes were found in clades Ia, Ie, II, and IIIb (4 of 4 individuals in wooD); *B*. *americanus* haplotypes in clade I were sampled from New York, while those in clades II and III were sampled from Missouri and Oklahoma. Five *B*. *woodhousii* haplotypes occurred in clade III. Twenty-five *B*. *houstonensis* included here, and in the statistical parsimony network below, were not analysed as part of the microsatellite dataset (these had haplotypes wooA and wooC).

The statistical parsimony network of 14 unique mtDNA haplotypes in 160 *B*. *houstonensis* is presented in Fig. 4. When constructed under a 95 % confidence criterion, two unconnected groups resulted; these two groups were forced together at 22 steps. Haplotypes wooA (n = 22) and wooC (n = 3) comprised one group. The other group had the following haplotypes: houA (n = 34), houB (n = 42), houC (n = 32), houD (n = 6), houE (n = 7), houF (n = 5), houG (n = 2), houH (n = 3), MF04876 (n = 1), MF05707 (n =

1), MF09351 (n = 1), and MF20073 (n = 1). Four private haplotypes were detected: MF04876 from BAN02p, MF05707 from BAN05p, MF09351 from BAN20t, and MF20073 from LEOp. Two haplotypes were detected in Austin County (houB and houF), ten in Bastrop Co. north (houA, houB, houC, houE, houG, MF04876, MF05707, MF09351, wooA, and wooC), six in Bastrop Co. south (houA, houB, houC, houE, houG, and houH), four in GLR p12 (houA, houC, houD, and houE), one in Colorado (houB), four in Lee (houA, houB, houD, and wooA), and two in Milam (houB and houF). Two dominant haplotypes were found in multiple geographic groups (houA in Bastrop Co. north, Bastrop Co. south, GLR p12, and Lee; houB in Austin, Bastrop Co. north, Bastrop Co. south, Lee, and Milam). Haplotypes houB and houC were found mostly in Bastrop Co. south (73.8 % and 93.8 %, respectively; these two haplotypes make up 83.6 % of all individuals sampled in these geographic groups).

Genetic clustering analyses

GENELAND analyses

Results from all GENELAND analyses are summarized in Tables 10 and 11. For the dataset including all *B. houstonensis* (n = 439) analysed using the spatial model in GENELAND (analysis A), the modal value for K was 4. Four individuals were unambiguously assigned to one cluster, cluster I; 196 were unambiguously assigned to another cluster, cluster N; 173 were unambiguously assigned to a third cluster, cluster S; and four were unambiguously assigned to a final cluster, cluster U. Only one cluster comprised Austin County, only one cluster comprised Colorado, and only one cluster comprised Milam (see

Fig. 1b). Out of 206 in Bastrop Co. north, 196 (95.1 %) were unambiguously assigned to cluster N. Out of 171 in Bastrop Co. south, 154 (90.1 %) were unambiguously assigned to cluster S. At GLR p12, which is geographically between Bastrop Co. north and Bastrop Co. south, all 39 individuals were assigned partial membership to clusters N and S. In Lee County, ten out of 11 (90.9 %) were unambiguously assigned to cluster S.

For the dataset including all *B. houstonensis* (*n* = 439) analysed without the spatial model in GENELAND (analysis B), the modal value for *K* was 3. One hundred ninety-seven individuals were unambiguously assigned to one cluster, cluster N; 167 were unambiguously assigned to another cluster, cluster S; and 62 were unambiguously assigned to a final cluster, cluster X. Most individuals from Austin County were assigned to multiple clusters, only one cluster comprised Colorado, and only one cluster comprised Milam. Out of 206 in Bastrop Co. north, 199 (96.6 %) were unambiguously assigned to cluster N. Out of 171 in Bastrop Co. south, 153 (89.5 %) were unambiguously assigned to cluster S; of those not assigned to cluster S, 17 were from BAS06p. At GLR p12, which is geographically between Bastrop Co. north and Bastrop Co. south, all 39 individuals were unambiguously assigned to cluster X. In Lee County, six out of 11 (54.5 %) were unambiguously assigned to cluster S. Seventy-six had different assignments when analysed without the spatial model. These individuals were from 12 sites: AUS01p (*n* = 1), AUS02s (1), AUS03p (2), BAN21t (1), BAN22t (1), BAN27s (4), BAPp (39), BAS06p (17), BAS10t (1), LEE01s (1), LEE03p (4), and MILs (4). In most of these cases, the assignments resulting from analysis without the spatial model were to cluster X. For example, at GLR p12, assignments changed from N+S to X, and at site BAS06p,

assignments changed from N to X. In Austin and Milam counties, where 'special' clusters were found using the spatial model (cluster U in Austin Co. and cluster I in Milam Co.), individuals were assigned to multiple clusters or to cluster X when analysed without the spatial model.

For the dataset including individuals for which there were no missing data (n = 72) analysed using the spatial model, the modal value for K was 3. Nine individuals were unambiguously assigned to one cluster, cluster I; 47 were assigned to another cluster, cluster N; and ten individuals were assigned to a final cluster, cluster S. Out of 55 in Bastrop Co. north, 46 (83.6 %) were unambiguously assigned to cluster N. Out of 15 in Bastrop Co. south, ten (66.7 %) were unambiguously assigned to cluster S.

Subset analyses, wherein only 20 individuals, randomly selected, from Bastrop and Lee counties were allowed, resulted in modal K values from 4 to 6 (mode = 4). In all ten subsets, individuals from Austin County were unambiguously assigned to cluster U, and individuals from Milam were unambiguously assigned to cluster I. In seven out of ten subsets, individuals from Colorado County were unambiguously assigned to cluster S. In six out of ten subsets, individuals from Bastrop Co. south were unambiguously assigned to cluster S.

For the dataset including individuals from only Bastrop and Lee counties (n = 427; analysis C) analysed using the spatial model, the modal value for K was 4. One hundred eighty-nine individuals were unambiguously assigned to one cluster, cluster N; 71 were unambiguously assigned to another cluster, cluster S_1; 75 were unambiguously assigned to a third cluster, cluster S_2; and 57 were unambiguously assigned to a final

cluster, cluster X (Fig. 1c). Out of 206 individuals in Bastrop Co. north, 189 (91.7 %) were unambiguously assigned to cluster N. Out of 171 in Bastrop Co. south, 69 (40.4 %) were unambiguously assigned to cluster S_1 and 62 (36.3 %) were unambiguously assigned to cluster S_2. Of the 40 not assigned to cluster S_1 nor cluster S_2, 17 were from BAS06p and they were unambiguously assigned to cluster X, and 23 were from sites BAS08p, BAS15p, and BAS18p and were assigned to multiple clusters, S_1 and S_2. At GLR p12, which is geographically between Bastrop Co. north and Bastrop Co. south, all 39 individuals were unambiguously assigned to cluster X. In Lee County, ten out of 11 (90.9 %) were unambiguously assigned to cluster S_2.

For the dataset including individuals from only Bastrop and Lee counties (n = 427; analysis D) analysed without the spatial model, the modal value for K was 3. One hundred ninety-six individuals were unambiguously assigned to one cluster, cluster N; 166 were unambiguously assigned to another cluster, cluster S; and 57 were unambiguously assigned to a final cluster, cluster X. Out of 206 in Bastrop Co. north, 196 (95.1 %) were unambiguously assigned to cluster N. Out of 171 in Bastrop Co. south, 153 (89.5 %) were unambiguously assigned to cluster S; of the 18 not assigned to cluster S, 17 were from BAS06p and they were unambiguously assigned to cluster X. At GLR p12, which is geographically between Bastrop Co. north and Bastrop Co. south, all 39 individuals were unambiguously assigned to cluster X. In Lee County, six out of 11 (54.5 %) were unambiguously assigned to cluster S_2.

Second-order analyses using the spatial model (analysis E), wherein only individuals with an assignment of cluster N from certain sites (GLR, the Musgrave

property, and Sandy Creek) in Bastrop Co. north were included (n = 195), or wherein only individuals with an assignment of cluster S from certain sites (BSP, Bluebonnet Headquarters, and the Jim Small property) in Bastrop Co. south were included (n = 154), resulted in modal K values of 1 and 2, respectively; that is, GENELAND detected only one cluster in Bastrop Co. north, while for Bastrop Co. south, GENELAND detected two clusters. Out of 154 in Bastrop Co. south, 79 (51.3 %) were unambiguously assigned to cluster S_1 and 62 (40.3 %) were unambiguously assigned to cluster S_2. The remaining 13 individuals were from sites BAS15p and BAS18p.

Second-order analyses without the spatial model (analysis F), wherein only individuals with an assignment of cluster N from certain sites (GLR, the Musgrave property, and Sandy Creek) in Bastrop Co. north were included (n = 195), or wherein only individuals with an assignment of cluster S from certain sites (BSP, Bluebonnet Headquarters, and the Jim Small property) in Bastrop Co. south were included (n = 154), resulted in modal K values of 1 and 2, respectively; that is, GENELAND detected only one cluster in Bastrop Co. north, while for Bastrop Co. south, GENELAND detected two clusters. Out of 154 in Bastrop Co. south, 74 (48.1 %) were unambiguously assigned to cluster S_1 and 70 (45.4 %) were unambiguously assigned to cluster S_2. The remaining ten individuals were from site BAS08p.

STRUCTURE analyses

Results from all STRUCTURE analyses are summarized in Tables 12 and 13. For the dataset including all *B. houstonensis* (n = 439; analysis G), the most likely number of

clusters was 2; all ad hoc measures of Evanno (2005) support this. The highest average log-likelihood for $K = 2$ was –13,505.1 and ΔK was 2,023.9. One hundred ninety-seven individuals were unambiguously assigned to one cluster, cluster N; 181 were unambiguously assigned to the other cluster, cluster S; and 61 were assigned partial membership to clusters N and S. Both clusters occurred in all counties except Austin Co. where only cluster N was present. Out of 206 in Bastrop Co. north, 166 (80.6 %) were unambiguously assigned to cluster N. Out of 171 in Bastrop Co. south, 138 (80.7 %) were unambiguously assigned to cluster S. At GLR p12, which is geographically between Bastrop Co. north and Bastrop Co. south, out of 39 individuals, 14 were assigned to N and 16 were assigned to S. In Colorado County, two (66.7 %) were unambiguously assigned to cluster S. In Milam County, three (75 %) were unambiguously assigned to cluster S.

For the dataset including individuals for which there were no missing data (n = 72), the most likely number of clusters was 3; all ad hoc measures of Evanno (2005) support this. The highest average log-likelihood for $K = 3$ was –2,454.8 and ΔK was 60.6. Twenty-one individuals were unambiguously assigned to one cluster, cluster N; 12 were unambiguously assigned to another cluster, cluster S; 18 were unambiguously assigned to a final cluster, cluster I; and 21 were assigned partial membership to multiple clusters. Out of 52 in Bastrop Co. north, 19 (36.5 %) were unambiguously assigned to cluster N and 16 (30.8 %) were unambiguously assigned to cluster I. Out of 15 in Bastrop Co. south, ten (66.7 %) were unambiguously assigned to cluster S.

Subset analyses, wherein only twenty individuals, randomly selected, from

Bastrop and Lee counties were allowed, resulted in *K* values from 2 to 7 (mode = 4). For most subsets, the ad hoc measures of Evanno (2005) supported the stated value of *K*; however, for some subsets, the measures conflicted with one another and the more biologically meaningful value of *K* was chosen. In seven out of ten subsets, individuals from Austin County were unambiguously assigned to cluster U. Individuals from Milam County were unambiguously assigned to cluster I in three out of ten subsets and to cluster S in another three subsets out of ten.

For the dataset including individuals from only Bastrop and Lee counties (*n* = 427; analysis H), the most likely number of clusters was 2; all ad hoc measures of Evanno (2005) support this. The highest average log-likelihood for *K* was –13,100.9 and Δ*K* was 979.2. One hundred ninety-seven individuals were unambiguously assigned to one cluster, cluster N; 176 were unambiguously assigned to the other cluster, cluster S; and 54 were assigned to both clusters. Out of 206 in Bastrop Co. north, 167 (81.1 %) were unambiguously assigned to cluster N. Out of 171 in Bastrop Co. south, 139 (81.3 %) were unambiguously assigned to cluster S. At GLR p12, which is geographically between Bastrop Co. north and Bastrop Co. south, out of 39 individuals, 14 were assigned to N and 16 were assigned to S. In Lee County, out of 11, 6 (54.5 %) were unambiguously assigned to cluster S.

Second-order analyses (analysis I), wherein only individuals with an assignment of cluster N from certain sites (GLR, the Musgrave property, and Sandy Creek) in Bastrop Co. north were included (*n* = 163), or wherein only individuals with an assignment of cluster S from certain sites (BSP, Bluebonnet Headquarters, and the Jim

Small property) in Bastrop Co. south were included (n = 135), resulted in an appropriate K value of 2 for each analysis; that is, STRUCTURE detected 2 clusters in Bastrop Co. north and 2 clusters in Bastrop Co. south. All ad hoc measures of Evanno (2005) support this for Bastrop Co. north and half of the measures support it for Bastrop Co. south. The other two measures, including ΔK, indicate that K = 5; however, K = 2 is more biologically meaningful, given other results and the large variances found for K = 5 (data not shown). The highest average log-likelihoods for K = 2 were –4,746.6 and –3,959.8, and ΔKs were 428.8 and 3.5, respectively. Individuals with multiple cluster memberships, resulting from analysis of all individuals (analysis G), were excluded from both analyses. Individuals with memberships in cluster S were excluded from the second-order analysis of Bastrop Co. north; individuals with memberships in cluster N were excluded from the second-order analysis of Bastrop Co. south. In Bastrop Co. north, 62 individuals (38.0 %) were unambiguously assigned to N_1 and 56 (34.4 %) were unambiguously assigned to cluster N_2. In Bastrop Co. south, 44 individuals (32.6 %) were unambiguously assigned to S_1 and 42 (31.1 %) were unambiguously assigned to cluster S_2.

When individuals from outside of Bastrop and Lee counties were excluded (analysis H), few assignments were different from the analysis of the original dataset (analysis G). Only 12 individuals at ten sites were assigned differently; in all 12 cases, a membership changed either from an unambiguous assignment to a partial assignment in multiple clusters or from a partial assignment in multiple clusters to an unambiguous assignment.

Genetic diversity analyses

Characteristics of genetic diversity are presented in Tables 14 and 15. In ten microsatellites, total number of alleles was 164. Across the nine clusters or groups described above (Table 15), number of alleles ranged from 7 to 132 and private alleles ranged from 0 to 29. Among loci, number of alleles ranged from 8 to 29, private alleles ranged from 2 to 10, and allelic richness ranged from 1.402 to 3.516. After sequential Bonferroni correction, only one locus (BC52.12) at one group ('I') significantly deviated from HWE. Loci BBR34-2 and BC52.10 were determined to be in LDE in all nine groups ($P = 0.00012$; 8100 permutations); however, the adjusted α-level was 0.000123, so this linkage was only just significant. No loci were in LDE when the groups identified by STRUCTURE were analysed (1800 permutations).

Pairwise F_{ST} values were calculated for multiple groups of sites. See Tables 16 and 17 for results among the four clusters identified by GENELAND ($F_{ST} = 0.035$-0.422) and for results among the nine groups identified via multiple methods ($F_{ST} = 0.046$-0.400). F_{ST} for Bastrop County vs. all others was 0.032 ($P < 0.0001$), for Austin County vs. all others was 0.199 ($P < 0.00001$), and for 'N' vs. 'S' was 0.045 ($P < 0.00001$). Pairwise F_{ST} values associated with Austin County were generally the highest (0.193-0.422, Table 16; 0.196-0.400, Table 17), while the lowest values were among the groups in Bastrop County (0.035, Table 16; 0.046-0.118, Table 17).

Mantel tests using either the microsatellite or mtDNA data indicated significant positive, but small, correlations between genetic distances and geographic distances (i.e., isolation-by-distance) for all analyses ($r = 0.0698$ to 0.1591; Table 18).

Migration rates

In the analysis of the entire *B*. *houstonensis* range, all ten BAYESASS runs converged on similar solutions for migration rates (data not shown). Migration rates from the best run are presented in Table 19; proportion of residents per group ranged 69.4 %-99.4 %. Standard deviations were mostly <0.05; seven (out of 361) were between 0.052 and 0.081. Among the 19 groups from across the entire range (see Table 6), migration rates were generally low; immigrants account for >10 % of the population in only three groups: BANeast from BANwest, BAS08p from BASs1, and LEE01s from LEE02,03. In the latter case, only one individual was collected from LEE01s, so it is impossible that 11.3 % of one individual was an immigrant; additionally, the individuals at LEE02p and LEE03p were all assigned as 'resident' to LEE01s at time 0, indicating that LEE01s and LEE02,03 were one group instead of two as identified a priori. Migration rates were asymmetric in the other two cases. Migration from BANwest to BANeast was 15.2 %, and from BANeast to BANwest it was 7 %. Migration from BASs1 to BAS08p was 10.8 %, and from BAS08p to BASs1 it was <0.1 %. Two hundred twenty-five out of 363 (61.98 %) males were residents, 14 out of 29 (48.28 %) females were residents, and 9 out of 26 (34.62 %) juveniles were residents.

While males were more likely than females to be 'resident' throughout the range (61.98 % vs. 48.28 %), these proportions were not significantly different according to the proportion test (H_0: proportion of males that were residents = proportion of females that were residents; $Z = 1.45 < 1.96$ so fail to reject H_0; 95 % CI = -0.003, 0.277). In contrast, the proportion test comparing adults with juveniles (60.97 % vs. 34.62 %) showed that

the proportion of adults that were 'resident' was significantly different from the proportion of juveniles that were 'resident' (H_0: proportion of adults that were residents = proportion of juveniles that were residents; $Z = 2.64 > 1.96$ so reject H_0; 95 % CI = 0.007, 0.519).

In the analysis of BANwest (Table 7), all ten BAYESASS runs converged on similar solutions for migration rates in five out of 256 combinations (in these five cases, nine out of ten runs converged on similar solutions; data not shown). Migration rates from the best run are presented in Table 20; proportion of residents per group ranged 69.4 %-93.6 %. Standard deviations were mostly <0.05; twelve (out of 256) were between 0.051 and 0.080. Migration rates were generally low; immigrants account for >10 % of the population in only one group: BAN06p from BAN04p. Migration rates were asymmetric for this pair of sites; migration from BAN04p to BAN06p was 16.7 %, and from BAN06p to BAN04p it was 0.5 %. Thirty-nine out of 123 (31.71 %) males were residents, 3 out of 19 (15.79 %) females were residents, and 1 out of 18 (5.56 %) juveniles were residents.

The proportion of males that were 'resident' was not significantly different from the proportion of females that were 'resident' in BANwest (H_0: proportion of males that were residents = proportion of females that were residents; $Z = 1.35 < 1.96$ so fail to reject H_0), but the 95 % CI indicated that the two groups were different (0.025, 0.277). Moreover, the proportion test comparing adults with juveniles (29.57 % vs. 5.56 %) showed that the proportion of adults that were 'resident' was significantly different from the proportion of juveniles that were 'resident' (H_0: proportion of adults that were

residents = proportion of juveniles that were residents; $Z = 2.17 > 1.96$ so reject H_0; 95 % CI = 0.097, 0.384).

AMOVA analyses

AMOVA results showed that most of the variance was within sites (65.12 %-92.67 %; Table 21). Whether individuals are grouped via STRUCTURE (Table 21 [A]), GENELAND (Table 21 [B, D]), or multiple methods (Table 21 [C and E]), the % total variance was around four. When individuals were partitioned into Austin County vs. all other counties, 19.10 % of the variance was between these two groups. Little partitioning among years within sites was found (3.36 %).

Discussion

Historic range and current distribution

When first described in 1953, *B. houstonensis* was known to occur in five counties (Sanders 1953): Austin, Burleson, Colorado, Harris, and Liberty. By 1970, it had been discovered in Bastrop and Ft. Bend counties (Brown 1971). By 1991, *B. houstonensis* had also been found in Lavaca, Leon, Milam, and Robertson counties (Yantis 1989, 1991). *Bufo houstonensis* had been thought to occur in Lee County for years (Yantis 1990, 1992; Yantis & Price 1993; Seal 1994) before it was recorded as present in 2001 (Forstner & Dixon 2001; Gaston et al. 2001).

Bufo houstonensis is now likely extirpated from Ft. Bend (last seen in 1965-7, Yantis 1992; Yantis & Price 1993), Harris (last seen in 1976, Yantis 1992; Yantis &

Price 1993), Lavaca (last seen in 1991, Forstner et al. 2008), and Liberty (last seen in 1950s, Yantis 1992; Yantis & Price 1993) counties. Recent surveys (2007-2008 breeding seasons) were performed in Anderson, Austin, Bastrop, Burleson, Colorado, Ft. Bend, Guadalupe, Harris, Henderson, Lavaca, Lee, Leon, Liberty, Limestone, Milam, Robertson, and Wilson counties (Forstner et al. 2007b; Forstner et al. 2008); toads were observed in Austin, Bastrop, Colorado, Lee, Leon, and Milam. Forstner et al. (2008) concluded that *B. houstonensis* was unlikely to continue to occur in Lee Co. and that very low numbers were present in Austin, Colorado, and Leon counties. No toads were observed in Burleson (last seen in 1990) and Robertson (last seen in 2000) counties (Forstner et al. 2007b; Forstner et al. 2008); while fewer surveys were performed in these counties than in other counties, it is possible that *B. houstonensis* might no longer occur in Burleson and Robertson. Currently, the largest numbers of *B. houstonensis* are found in Bastrop County (Michael R. J. Forstner, personal communication).

Literature and museum record searches indicate that *B. houstonensis* has been observed only once for three counties: Ft. Bend, Lavaca, and Liberty (see Table 1). Observations in Ft. Bend and Liberty might have been based only on hearing a mating call (James R. Dixon & Jim Yantis, personal communication to Michael R. J. Forstner), and, unfortunately, no specimens exist for these counties. Additionally, the literature museum record searches found specimens from outside the known range (see Appendix B): Brazos, Freestone (Yantis 1990), Houston, and Travis. These specimens need to be reviewed to ensure their proper identification. For example, the Freestone Co. specimen was collected outside the breeding season (on 16 Oct 1990), and the collector (J. H.

Yantis) returned to the site during the breeding season in 1991 but found only *B*. *woodhousii* (James H. Yantis, personal communication).

Phylogeny and haplotype network

Currently up to ten species comprise the *Bufo americanus* species group: *B*. *americanus*, *B*. *baxteri*, *B*. *californicus*, *B*. *fowleri*, *B*. *hemiophrys*, *B*. *houstonensis*, *B*. *microscaphus*, *B*. *terrestris*, *B*. *velatus*, and *B*. *woodhousii* (Masta et al. 2002; Pauly et al. 2004). *Bufo houstonensis* has been placed in this group since its description (Sanders 1953). Many characteristics support its placement here: cranial features (Sanders 1953), egg string morphology (Sanders 1953), mating call features (Blair 1956, 1962, 1963), genetic compatibility (Blair 1962, 1963), conventional morphology (Blair 1962, 1963), osteology (Tihen 1962; Martin 1973), parotoid venom (Blair 1963), ecological and geographical evidence (Blair 1963), karyology (Sanders & Cross 1964), blood proteins (Guttman 1969), allozymes (Thomas & Dessauer 1982; Hillis & Price 1993), and molecular phylogenetics (Pauly et al. 2004; Goebel et al. 2009).

Results presented here (Fig. 2) support the placement of *B*. *houstonensis* in the *B*. *americanus* species group; moreover, they indicate a close relationship with *B*. *americanus* as suggested by previous authors (Pauly et al. 2004; Goebel et al. 2009). As in other studies (Masta et al. 2002; Pauly et al. 2004), some taxa share haplotypes (Fig. 2, both *B*. *americanus* and *B*. *houstonensis* occurred in clade III with *B*. *woodhousii*) or were paraphyletic (Fig. 2, *B*. *americanus* occurred in clades I, II, and III). Haplotype sharing might be explained by sampling locality (e.g., Masta et al. 2002). All individuals

that shared haplotypes with *B*. *woodhousii* were sampled in areas of species range overlap. The *B*. *houstonensis* in clade III were sampled in Bastrop County where *B*. *woodhousii* is found, and the *B*. *americanus* in clade III were sampled in Missouri and Oklahoma where *B*. *woodhousii* is also found (Table 4). Of course, hybridization events might also explain these haplotypic patterns (but see Chapter 4). For the apparent paraphyly of *B*. *americanus* (Fig. 2), it is possible that it is truly paraphyletic and *B*. *houstonensis* is nested within, but it is also possible that the marker used here (~500 bp mtDNA D-loop sequence) is too invariant or too short to assess the close relationship between the two taxa. Indeed, other markers, such as microsatellites or SNPs, might provide more discerning evidence to tease apart the relationships within the *B*. *americanus* species group (Hillis & Price 1993).

Fourteen haplotypes were recovered in *B*. *houstonensis* (Fig. 2). Uncorrected pairwise distances ranged from 0.002 to 0.051 with an average of 0.021 in *B*. *houstonensis*. Excluding haplotypes wooA and wooC, the range and average were 0.002 to 0.045 and 0.016. Average distances in other taxa were 0.026 for *B*. *americanus* (five haplotypes) and 0.009 for *B*. *woodhousii* (6 haplotypes). *Bufo houstonensis* was sampled over the smallest geographic area (Tables 2, 4) but did not have the lowest number of haplotypes nor the lowest average pairwise distance. However, sample sizes for the three taxa were unequal (8, 160, 20 for *B*. *americanus*, *B*, *houstonensis*, and *B*. *woodhousii* respectively) and this might explain the differences among average distances or among number of haplotypes. Nevertheless, if the Hill County *B*. *woodhousii* (n = 15, two haplotypes, all sampled from one locality in this county) are compared to the GLR p12 *B*.

houstonensis (*n* = 11, four haplotypes), one haplotype per 7.5 *B*. *woodhousii* is found but one haplotype per 2.75 *B*. *houstonensis* is found. This evidence indicates that *B*. *houstonensis* is more diverse mitochondrially, due either to ancestral polymorphism or to its patchy and/or restricted occurrence on the landscape historically.

Bufo houstonensis is generally believed to be a post-Pleistocene relict derived from *B*. *americanus* less than 10,000 years ago (Blair 1963, 1965, 1972). Uncorrected sequence pairwise divergences between species, including the outgroup *B*. *cognatus*, ranged from 2.666 % to 17.509 %; within the *B*. *americanus* species group divergences ranged up to 6.827 % (Table 22). *Bufo houstonensis* is most closely related to *B*. *americanus* and *B*. *woodhousii* (2.666 % and 3.822 % respectively). Using a rate of 1.644 % divergence per lineage (3.288 % pairwise) per million years for the D-loop (Stöck et al. 2006), the date of divergence for *B*. *americanus* and *B*. *woodhousii* is estimated at 1.142 to 0.229 million years ago (mya), and divergence between *B*. *americanus* and *B*. *houstonensis* is estimated at 1.314 to 0.171 mya (Table 22). While the use of molecular clocks is contested at best (Maxson 1984; Hillis 1987; Moritz et al. 1987; Holder & Lewis 2003; Stöck et al. 2006; Goebel et al. 2009), if these estimated divergence dates are even within an order of magnitude of the actual dates, then *B*. *houstonensis* diverged far earlier than the proposed date of 10,000 years ago and likely existed during and perhaps before the Last Glacial Maximum.

Of the 14 *B*. *houstonensis* haplotypes, four were particularly frequent: houA, houB, houC, and wooA (Fig. 4). The most geographically widespread haplotypes were houB (Austin, Bastrop, Colorado, Lee, and Milam counties) and houF (Austin, Colorado,

and Milam counties). Within Bastrop County a trend was apparent among the four most frequent haplotypes: houA and wooA were more likely found in the north subgroup while houB and houC were more likely found in the south subgroup (Fig. 4). Furthermore, haplotypes found in Austin, Colorado, and Milam counties appear to be more closely related to haplotypes found in subgroup Bastrop Co. south than to those found in subgroup Bastrop Co. north (Fig. 4, houB and houF). Congruent with other studies (Hillis & Price 1993; Masta et al. 2002; Pauly et al. 2004), there is evidence for hybridization among species and for reticulate relationships among populations of *B. houstonensis* (Figs. 2, 4). Evidence from both the phylogeny and the statistical parsimony network do not rebut *B. houstonensis* as a distinct lineage.

Number of populations in B. houstonensis

This study is the first to assess population genetic structure at a landscape or a fine scale in *B. houstonensis*. Ten polymorphic loci in 439 samples from 57 sites in five counties (Table 2) were analysed using the genetic clustering software GENELAND and STRUCTURE to determine the number of populations. Oversampling (97.3 % of all individuals were sampled in Bastrop and Lee counties) did not bias GENELAND analyses, but STRUCTURE was far more influenced by sampling bias (Tables 9, 11). For example, cluster U in Austin County (found in analysis A) was recovered in all ten subsets using GENELAND, but only seven subsets in STRUCTURE; cluster I in Milam County (found in analysis A) was recovered in all ten subsets using GENELAND, but only three subsets in STRUCTURE; and cluster S in Colorado County (found in analysis A) was recovered in seven subsets

using GENELAND, but only two subsets in STRUCTURE. Whether missing data biased the results is more difficult to interpret because all individuals from Austin, Colorado, and Leon counties had some missing data and thus could not be included in the analysis (Tables 9, 11). Even so, assignment patterns parallel those found in analysis A: most individuals from Bastrop Co. north were assigned to cluster N and most from Bastrop Co. south were assigned to cluster S. Some of the missing data skew might be related to locus BC52.12 amplifying in only 171 out of 439 individuals (Table 15). Genotyping this locus was most successful for individuals from Bastrop Co. north (60.7 %, group N in Table 15), western Bastrop Co. south (53.3 %, group S_2), Milam County (75 %, group I). BC52.12 did not amplify for any individuals in groups BAS06p, COLs, LEOp, and U. Few individuals could be scored in GLR p12 (5.1 %, group BAPp) or eastern Bastrop Co. south (9.9 %, group S_1). Locus BC52.03 also had many missing data (51.3 % individuals amplified). However, the geographic pattern is less clear: 53.8 % in BAPp, 58.8 % in BAS06p, 33.3 % in COLs, 0 % in LEOp, 50 % in I, 70.9 % in N, 16.9 % in S_1, 53.3 % in S_2, and 0 % in U. In the end, missing data were not determined to influence the results enough to warrant excluding any loci from analyses.

Results from analyses A-I suggest that there are nine clusters at possibly different levels of divergence (see Figs. 1b, 1c). Six of these clusters are cluster I (in Milam County), cluster N (mainly in Bastrop Co. north), cluster S_1 (mainly in eastern Bastrop Co. south), cluster S_2 (mainly in western Bastrop Co. south), cluster U (in Austin County), a cluster at GLR p12 (= site BAPp), and a final cluster at site BAS06p. Although Colorado County grouped with cluster S and Leon County grouped with cluster

N, these sites are most likely independent from their assigned clusters given their geographical distance (~80 km and ~140 km respectively). And while sites BAPp and BAS06p were assigned to cluster X under analysis C, they were assigned dissimilarly under analysis A.

Genetic diversity

Microsatellite loci were used to assess several population diversity and structure measures. Only one group for one locus (group I, locus BC52.12) significantly deviated from HWE after sequential Bonferroni correction (Tables 14, 15). Only two loci (BBR34-2 and BC52.10) were found to be in LDE but they were only just significant (P = 0.00012 for α = 0.000123) so these loci were not excluded from analyses. Private alleles were found in all but two of the nine groups (COLs and U). Allelic richness was similar across the groups that could be evaluated but was different across loci (Table 15).

Pairwise F_{ST} values among the nine groups indicate high levels of differentiation among groups >85 km apart (F_{ST} >0.25, BAPp-U, BAS06p-I, BAS06p-U, and I-U, Table 17). Among the geographically proximate groups N, S_1, and S_2, differentiation was low to moderate (F_{ST} = 0.046-0.081); specifically, N was more closely related to S_2, and S_1 was more closely related to S_2. However, there were also high levels differentiation at distances as little as 4 km (F_{ST} = 0.118 for BAS06p-S_1, see also Fig. 1c). This result, increasing F_{ST} with increasing geographic distance but some high F_{ST} at smaller distances, fits with other studies on pond-breeding bufonids (e.g., Rowe et al. 2000; Brede & Beebee 2004; Martínez-Solano & González 2008).

Mantel tests using microsatellite data indicate little isolation-by-distance (0.0698, Table 18); given the high F_{ST} at the landscape scale, this result is surprising. Higher levels of isolation-by-distance were found using mitochondrial haplotypes (0.1591) but this is expected because mtDNA by definition has a smaller effective population size and thereby smaller sample size compared to microsatellite loci used here.

Migration

Very low levels of migration occur over the entire range of extant *B*. *houstonensis* (Table 19). At distances of >50 km, little migration is expected (e.g., among Austin, Leon, and Milam counties, see Fig. 1b). But in some cases, little migration was seen even at distances at ~4 km; for example, the easternmost pond in Bastrop Co. south, site BAS06p (see Fig. 1c), is situated ~4 km from the nearest pond yet 98.2 % of the individuals sampled here were residents at this pond. Similarly, BAPp is <2 km from the nearest pond but 93.4 % of the individuals sampled here were residents. Typically though, some migration occurred at these distances, like at site BAS08p where ~10 % of individuals immigrated from 2.5 to 4.5 km away in BASs1 or at BANeast where 15.2 % immigrated from 1 to 3 km away in BANwest (see Table 6 for definitions). The highest migration rate in Bastrop Co. north was west to east (BANwest to BANeast, Table 19); the simplest explanation here is that toads moved downslope along a tributary of Alum Creek running NW-SE (see also Fig. 1c). *Bufo houstonensis* has been shown to utilize a 5 m area next to a drainage when moving overland (221 m travelled, Swannack 2007). When migration occurred in Bastrop Co. south, toads moved east to west (BASs1 to BAS08p, Table 19).

One possible explanation of movement east to west in this part of Bastrop County is that in dry years toads might move towards more permanent water bodies, like Lake Bastrop (~3.7 km^2 area) which lies just west of the westernmost sampling sites in Bastrop Co. south in Fig. 1c. Although the direction of movement is upslope, the maximum elevational change in this area is only ~40 m.

Movement of *B. houstonensis* has not been tracked outside the breeding season. But, a multi-year trapping study along with tracking studies have shown that typical adults likely remain within 200 m of their breeding pond during their lifetime (Forstner & Swannack 2004; Swannack et al. 2006; Swannack 2007). Juveniles are probably the dispersal life-stage in *B. houstonensis*, as they are in other bufonids like *B. bufo*, *B. calamita*, and *B. fowleri* (Breden 1987; Scribner et al. 1997; Sinsch 1997). Hillis et al. (1984) observed juvenile *B. houstonensis* up to 100 m away from a pond in gulleys leading to ponds. In my study, the proportion of residents in adults was significantly different from juveniles ($Z = 2.64$); that is, juveniles either moved more frequently or moved farther than adults. The longest straight-line distance travelled by an adult male *B. houstonensis* in 24 hr is 500 m (Price 1992) and in 4 weeks is ~2000 m (Forstner et al. 2003; Price 2003). Fewer female accounts exist, but the longest distance recorded is 675 m in ~2 weeks (Price 1992). Comparable long-range dispersal, or even farther, has been documented in other bufonids: 4 km for *B. americanus* (Maynard 1934), 6 km in 4 yr for *B. boreas* (Muths et al. 2003), 1.6 km in several weeks for *B. bufo* (Sinsch 1988), 2.6 km in a breeding season for *B. calamita* (Sinsch 1992), and 2 km in 2 yr for *B. fowleri* (Breden 1987). In *B. quercicus*, Greenberg & Tanner (2005) found that very few toads

move between breeding ponds and when they did it was only a distance of about 132 m. In my study, the proportion of male residents was higher than females (although the difference was not significant) which means that females might move more often or farther across the landscape than males. Females tend to roam farther than males in *B. boreas* (Muths 2003; Bull 2006), in *B. calamita* (Sinsch 1992), and in *B. japonicus* (Kusano et al. 1995). Typical movement distances and long-distance dispersals attainable by *B. houstonensis* correspond to the migration rates found here. Migration rates were higher at <4 km (e.g., BANwest to BANeast, Table 19), but some movements probably occurred at greater distances (e.g., 0.056, BANwest to BANnorth, ~5 km, Table 19).

Analysis of molecular variance

Contrary to genetic clustering analyses, pairwise F_{ST} values, and migration rates, AMOVAs strongly indicate that, regardless of how populations or groups are delineated, little variation (3.48 % to 4.80 %) was explained at this level, and most of the variation was within sites (89.21 %-92.67 %, Table 21 [A-E]). However, 19.10 % of the total variation was explained (Table 21 [G]) when groupings were sites in Austin County (= group U in Tables 15, 17) and sites in all others, indicating that *B. houstonensis* in Austin County is very different from all other *B. houstonensis*.

Summary of evidence for nine populations

In addition to the genetic clustering findings, other lines of evidence were identified that support the hypothesis that there are nine populations of *B. houstonensis* (BAPp, BAS06p, COLs, LEOp, MILs, N, S_1, S_2, and U). High F_{ST} values and low migration rates

(Tables 17, 19) strengthen the inference that BAS06p and BAPp are distinct from other groups in Bastrop County (F_{ST} = 0.080-0.118, migration rates <0.03). Other groups in Bastrop County (N, S_1, and S_2) were less differentiated and some had higher migration rates (Tables 17, 19). However, most migration occurred within each of these groups (see boxes in Table 19); for example, highest immigration to BANeast was from BANwest and highest emigration from BANeast was to BANwest and BANnorth. The broader north and south division within Bastrop County is corroborated by mitochondrial haplotype data: haplotypes houA and wooA were found more often in Bastrop Co. north than in Bastrop Co. south while houB and houC were found more frequently in the south than in the north (Fig. 4).

Outside of Bastrop County, a unique mitochondrial haplotype (MF20073; Figs. 2, 4) establishes that LEOp in Leon County might be a separate lineage; moreover, though LEOp was assigned to cluster N, the LEOp-N F_{ST} was 0.204 (Table 17). Pairwise F_{ST} for MILs in Milam County were also quite high (0.143-0.400, average 0.209, Table 17) which indicate high levels of differentiation of MILs from other groups. The highest F_{ST} were found in Austin County (0.196-0.400, average 0.273, Table 17) indicating that this population is the most diverged. Plus, the AMOVA model that explained the highest amount of variation was when Austin County was set apart from all other sites (19.10 %, Table 21 [E]).

Provenance of extant toads in Colorado County

Individuals from group COLs (in Colorado County) were assigned to the same cluster as

many from Bastrop Co. south (cluster S), but COLs had moderate to high F_{ST} values with groups in Bastrop County (0.077-0.118, Table 17). Site COLs and sites in Austin County are ~13 km apart (Fig. 1b). Unexpectedly, individuals from COLs were not assigned to cluster U (= group U in Austin County). COLs also had low migration rates with group U (0.003 and 0.012, Table 19) and a high F_{ST} with group U (0.339, Table 17). One possible reason for these results involves the translocation program conducted by the Houston Zoo in the 1980s as part of the Houston Toad Recovery Plan (Quinn 1980; Quinn et al. 1984; Potter et al. 1984; Quinn et al. 1987). *Bufo houstonensis* was collected from Bastrop County, reared at the Houston Zoo, and then translocated to the Attwater Prairie Chicken National Wildlife Refuge (APCNWR, ~30 km SE of the 2007 sample site used in my study) in Colorado County. Over five years, ~400,000 eggs, ~7,000 metamorphs, and 62 adults were released at APCNWR. Measuring success of the program is difficult because budgetary constraints allowed few return visits to survey APCNWR from 1987 onward (Quinn et al. 1987) but Dodd & Seigel (1991) cite that no new populations had been successfully established as of 1991. Yet, it is known that *B. houstonensis* bred in 1985 (a developing egg string was found) and called in two years (one male in 1984 and seven in 1986) at sites near the San Bernard River which abuts the refuge (Quinn et al. 1984; Quinn et al. 1987).

The collection sites for the translocation program are identical to or are <2 km from sites sampled for this study in Bastrop Co. south, specifically in an area where most individuals were assigned to cluster S_1 (see Fig. 1c). According to pairwise F_{ST} values, out of the other eight groups, COLs was least differentiated from S_1 (Table 17). And, the

highest immigration rates into Colorado County were from S_2 and S_1 (0.036 and 0.027 respectively). Because the San Bernard River is close to both APCNWR and the 2007 sample site in Colorado County (~3 km from the river), it is feasible that toads and their descendants travelled along the river northward from APCNWR over the past 20 years and the results presented here characterize that movement.

Conservation management implications

Units for conservation

Data presented here do not fit the criteria for evolutionary significant units (ESUs) sensu Moritz (1994) because no mtDNA reciprocal monophyly exists for the nine groups described above. Debate continues over which definition works best (for a review see Fraser & Bernatchez 2001), but for my study, management unit (MU) sensu Moritz (1994), where significant divergence in allele frequencies exists but reciprocal monophyly of mtDNA alleles is not necessary, seems most appropriate. In extant *B. houstonensis* there are nine MUs, and they correspond to the nine groups described above: five in Bastrop County (BAPp, BAS06p, N, S_1, S_2), Austin County (group U), Colorado County (COLs), Leon County (LEOp), and Milam County (MILs, group I).

While little gene flow was apparent at distances >4 km, some mtDNA haplotypes (houB and houF in Austin and Milam counties) and some microsatellite alleles (Austin and Colorado counties have no private alleles) are found throughout the range. And, the overall diversity in *B. houstonensis* is high; 14 mtDNA haplotypes were recovered (Fig. 4, four were singletons), and number of alleles per locus (8-29, Table 15), average

alleles/locus/population (5.73), and average expected heterozygosity (0.624) are comparable to or higher than in a variety of other anurans (see Table 3 in Ficetola et al. 2007). In fact, average alleles/locus/population and expected heterozygosity are higher than those for another declining yet more widespread bufonid, *Bufo calamita* (3.3, 0.388), and for an abundant and widespread bufonid, *Bufo bufo* (5.1, 0.579) (Ficetola et al. 2007). But because *B. houstonensis* has low vagility (Swannack 2007) and gene flow is low (i.e., connectivity appears to be minimal, data from this study), how has this diversity been maintained over the entire range? One answer may lie in the age of the species: *B. houstonensis* is potentially hundreds of thousands, or at least tens of thousands, of years old (Table 22). Over that period of time, novel haplotypes and alleles were created, and census sizes and connectivity among populations were probably greater than in recent decades. For example, Harris County populations were large around 1950 but declined rapidly until that last toad was seen in 1976 (Yantis 1992; Yantis & Price 1993). If toad populations were larger and more common historically than in the last century, then gene flow was possible throughout at least the northern part of the range. A relatively continuous band of deep sandy soils associated with *B. houstonensis* occurs from Bastrop County through Lee, Burleson, Milam, and Robertson counties to Leon County (soils derived from Carrizo, Queen City, and Sparta geologic formations, see Map 2 in Price 1990b). *Bufo houstonensis* is typically found in pine-hardwood forest or post oak woods/forest (for a detailed habitat description see Yantis 1990) which used to be common over the same areas as these deep sands (Brown 1975; GIS Lab at TPWD 1984). Consequently, populations of *B. houstonensis* could have occurred throughout the sandy

wooded area in close enough proximity to each other to allow even a little gene flow among them so that few populations were in complete isolation. Nonetheless, current populations are well separated from each other.

While diversity is high throughout the range, it is within an MU too, which bodes well for genetic management in this species. Number of alleles per locus was 0.7-13.2, expected heterozygosities were 0.588 to 1.000, number of private alleles was 1-29 (two MUs had no private alleles), and number of haplotypes was 1-10 for the nine MUs. This is likely a carryover of the range-wide diversity, but has greater impact on conservation strategies.

Threats to *B. houstonensis*

The greatest threat to *B. houstonensis* is low population size. Fewer than 200 toads are believed to be alive throughout the range (Michael R. J. Forstner, personal communication). Effective population sizes are almost always smaller than census population sizes in anurans (Easteal 1985; Dodd & Seigel 1991; Waldman et al. 1992; Scribner et al. 1994). The functional sex ratio in *B. houstonensis* is male-biased and was estimated to be 5.5M:1F (Swannack & Forstner 2007), thus the effective population size for all *B. houstonensis* may be as low as 70 (33 females + 33 males, if all females breed). It may be even lower if females are <2 years old because some females reach sexual maturity after two years (Quinn & Mengden 1984) or if chorus sizes are too small to attract females to breeding sites (Gaston et al. In review). Females appear to roam more than males. After breeding, females moved at least 50 m from the pond within two days

whereas males stayed longer near the pond (Swannack 2007). Females in other bufonid species exhibit similar tendencies (Sinsch 1992; Kusano et al. 1995; Muths 2003; Bull 2006). Ultimately, the few female *B*. *houstonensis* that are alive are moving either more often or greater distances than the more plentiful males, and in so doing may have higher mortality through predation (Potter et al. 1984; Freed & Neitman 1988; McHenry et al. In review) and road traffic (Price 1990a; Gaston et al. 2001).

Low numbers are likely consequent of habitat fragmentation/alteration and drought, although both have negative impacts even if population sizes are larger (Potter et al. 1984). Extirpation in Harris and Ft. Bend counties has been charged to both habitat change over many decades and severe drought during the 1950s (Potter et al. 1984; Brown 1994). And a drought beginning in the mid-1990s lowered numbers of toads in Bastrop State Park, where census numbers have usually been high (Price 2003). Southeast-central Texas is still in the midst of this drought (Forstner et al. 2007a). Habitat fragmentation or alteration (including urbanization and conversion to pasture or agriculture) is a chief direct threat (Potter et al. 1984; USFWS 2001). Habitat for *B*. *houstonensis* can be categorized as such: breeding and nursery habitat, occupied habitat, and dispersal habitat. Within the appropriate canopy and soil conditions, toads breed in usually small natural or artificial water bodies, preferring ephemeral pools and puddles to permanent bodies (Thomas & Potter 1975; Potter et al. 1984), where tadpoles remain before emerging as metamorphs 15-100 days later (Hillis et al. 1984; Quinn & Mengden 1984). Metamorphs stay within 3-5 m of the water body for five days and disperse up to 35 m away by 30 days (Greuter 2004). Occupied habitat is a breeding pond and the 200

m of adjacent upland where adults are most commonly found (Swannack 2007). Finally, dispersal habitat encompasses the corridors through which unidirectional juvenile or adult movement takes place. Drainages are the most likely corridor route for juveniles or adults, because first they are wet, but also because migration rates presented here indicate they are used (BANwest to BANeast, discussed above), Hillis et al. (1984) observed adults and juveniles using gulleys leading to ponds, and drainages were shown to be used by adults through telemetry (Swannack 2007). All three types of habitat must be protected to allow breeding, recruitment of juveniles into neighboring sites, and rescue of extinct sites (for a review of amphibian dispersal and migration processes see Semlitsch 2008). Due to the complexities of the life-cycle and habitat-use, habitat fragmentation is a primary concern.

Hybridization resulting in fertile offspring occurs between *B*. *houstonensis* and sympatric congeners, *B*. *nebulifer* and *B*. *woodhousii*, (Blair 1963; Brown 1971; Hillis et al. 1984) and is thought to be a consequence of habitat alteration (Brown 1971). Its impact as a threat is minimal (Brown & Thomas 1982; Hillis et al. 1984) in part due to the scarcity of *B*. *woodhousii* in the area of Bastrop (Brown 1971). However, due to increased habitat alteration, especially clearing of woods and forests, opportunities for hybridization events may increase: *B*. *nebulifer* occupies a wide variety of habitats including disturbed sites, *B*. *woodhousii* prefers open habitats (Brown 1971; Hillis et al. 1984), and both species breed in temporary and permanent water (Thornton 1955). Hybridization in *B*. *houstonensis* was investigated and is presented in Chapter 4.

Other potential threats include red imported fire ants (*Solenopsis invicta*, Freed &

Neitman 1988), bullfrogs (*Rana catesbeiana*, McHenry et al. In review), disease, and catastrophic fire (for more details on threats see Seal 1994). Chytrid fungus, *Batrachochytrium dendrobatidis*, was recently documented in *B*. *houstonensis* in Bastrop County (Forstner et al. 2007a); samples (most were samples used in my study) from 2001-2006 were tested but only those from 2006 were positive for the fungus (BAS01p, BAS07p, BAS09p, and BAS18p). However, samples of Bastrop County *B*. *nebulifer* were positive from 2001, 2004, and 2006 (Dittmar Hahn & James P. Gaertner, personal communication). Symptoms and pathogenicity in *B*. *houstonensis* are not known (for a review of chytrid fungi see Berger et al. 1999).

Future management strategies

Foremost, known populations should be monitored/surveyed every year. Numbers of toads are now so low that local extinctions are very probable, and if managers do not know that local extinctions have occurred, then conservation strategies will be ineffective. Secondly, increasing the numbers of toads must be achieved. Estimates of juvenile survival are between 0.0075 and 0.015 (Greuter 2004; Swannack et al. 2009), and estimates of survival of juveniles to adulthood are an order of magnitude higher (0.15-0.21, Swannack et al. 2009). As suggested by Swannack et al. (2009), conservation efforts towards improving juvenile survival will be well placed. Accordingly, supplementation programs, wherein individuals are added to an existing population (Seal 1994), should be chosen over reintroductions or translocations. Supplementation of individuals into their native population does not result in outbreeding depression, a

reduction in fitness in hybrid individuals (including individuals resulting from a mating between two intraspecific populations) relative to the parental types (Allendorf et al. 2001), which may be a problem in reintroductions or translocations. Local adaptations to environmental conditions that exist between populations may be broken down by translocating individuals from one population to another. In *B. houstonensis* this might occur if individuals from Bastrop County are introduced, say, into Austin County. Supplementation also avoids admixture of genetically distinct groups which could result in loss of diversity; again, the MU in Austin County is an example. A headstarting program, one type of supplementation designed to increase juvenile survivorship, was begun in 2007 (Forstner et al. 2007a). Eggs collected from the wild were reared at the Houston Zoo and then the juveniles were released into their natal ponds; juvenile survivorship was estimated near 40 %, more than 25 times the estimated value in the wild (Forstner et al. 2007a). Headstarting appears to work very well in *B. houstonensis* and could be key in conserving multiple MUs.

In addition to monitoring and increasing population sizes, conservation of all three *B. houstonensis* habitat types is crucial. Dispersal routes and distances have not been directly measured, but evidence for population connectivity within 4 km does exist (data from my study) and females probably roam more than males. Thus, in addition to breeding habitat and occupied habitat directly around a pond, corridors for dispersal between breeding sites must be protected. As for many other amphibian species, how chytridiomycosis affects *B. houstonensis* needs to be determined.

Protecting all nine MUs is not feasible due to budgetary constraints; moreover, it

may not be necessary. Toads now found in Colorado County are very likely descendants of the 1980s translocation program, and may represent a threat to toads in Austin County. If the descendants make their way into Austin County and the toads there cannot compete, then the diversity held in Austin County may be eliminated. *Bufo houstonensis* likely had greater connectivity in the past, evidenced by widespread occurrences of mtDNA haplotypes, high allelic diversity throughout the range, and lack of unique haplotypes in Austin County even though allele frequencies indicate it is very diverged. If *B. houstonensis* is to retain its natural historical levels of genetic variation, then protection of populations in Bastrop County and Austin County are necessary, as well as protection and restoration of dispersal corridors across the range.

Ultimately, conservation managers must involve the public throughout the range of *B. houstonensis*, as they have in Bastrop County. Most *B. houstonensis* occur on private land in Bastrop County, and managers have enlisted the help of private landowners to the benefit of the toads and the landowners themselves. In other counties, *B. houstonensis* also occurs primarily on private land, and similar outreach programs should be attempted as quickly as possible before more extirpations occur.

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Parks and Wildlife Department, Austin TX

Table 1 Number of *Bufo houstonensis* recorded per year by county

	Northern counties						Reference
	Bastrop	Lee	Burleson	Milam	Robertson	Leon	
1949							
1950			2*				Sanders 1953
1951	1*						
1952							
1956							
1958							
1959							
1960							
1963-4	42*						Brown 1971
1965-7	<300*		3				Brown 1971
1968	1*						
1971	3*						
1974-8							
1974	10s		2 (≥1)				Brown 1975; Thomas 1977
1975	50 (≥1)		10-20 (≥1)		2*		Thomas 1977
1976	100s (≥1)		<20 (≥1)				Thomas 1977; Potter et al. 1984
1977	>1 (≥1)	0 (≥1)	>1 (≥1)				Thomas 1977
1978	83*		0 (1)				Dixon 1983
1979	81*		0 (1)				Dixon 1983
1980	52*		0 (1)				Dixon 1983
1981	>1500*		0 (1)				Dixon 1983; Hillis et al. 1984
1982	≥215*		0 (≥1)				Dixon 1983; Jacobson 1989
1983	25*		4 (≥38)				Dixon 1983
1987	1*			1*			
1988	24*						
1989	8* (4)[a]		5* (20)	1 (≥4)	1* (≥4)	43* (≥3)	Yantis 1989
1990[b]	>300*		1 (90)	0 (30)		≥40* (35)	Price 1990b; Yantis 1990; Price 2003
1991	>400*		0 (≥1)			≥11*	Yantis 1991; Price 2003
1992	292*		0 (≥1)		>in 1991 (≥1)	>11 (≥1)	Yantis 1992; Yantis & Price 1993; Price 2003
1993	>250*						Thomas & Allen 1997; Price 2003
1994	>200						Price 2003
1995	>400						Price 2003
1996	>150	>5					Kuhl 1997; Price 2003
1997	>175	>30					Kuhl 1997; Price 2003
1998	>100						Price 2003
1999	>175						Price 2003
2000	>50 (22)	>100 (25)			1*		Forstner & Dixon 2000; Price 2003
2001	>100 (20)	>100* (>100)					Forstner & Dixon 2001; Price 2003
2002	>100 (92)	<15 (>100)					Forstner 2002; Price 2003
2003	≥200* (92)	0 (2)					Forstner 2003
2004	>45* (24)	1 (5)					Forstner & Swannack 2004
2005	127* (24)	1 (4)				0 (6)	Forstner 2006
2006	55 (39)	0 (18)				1 (5)	Forstner 2006
2007	118 (39)	1 (26)	0 (9)	>30 (22)	0 (12)	1 (9)	Forstner et al. 2007b
2008	94 (38)	0 (19)	0 (3)	2 (21)	0 (2)	>10 (9)	Forstner et al. 2008

Table 1 continued

	Southern counties						Reference
	Lavaca	Colorado	Austin	Ft. Bend	Harris	Liberty	
1949					66		Potter et al. 1984
1950					3*		Sanders 1953
1951					2*		
1952		9*	9*		≥40*	1	Sanders 1953
1956		1					Blair 1956
1958					5*		
1959					8*		Bragg 1960
1960					2		Kennedy 1961
1963-4					3		Sanders & Cross 1964
1965-7		0		1	3		Brown 1971
1968							
1971							
1974-8					2		Potter et al. 1984
1974							
1975							
1976					1 (≥2)		Thomas 1977; Potter et al. 1984
1977							
1978							
1979							
1980							
1981							
1982		1*					
1983							
1987							
1988							
1989		0 (9)	0 (3)		0 (3)	0 (10)	Yantis 1989
1990[b]		≥2* (468)	7* (41)	0 (415)	0 (15)		Yantis 1990
1991	7* (≥1)						Yantis 1991
1992	0 (≥1)				0 (≥1)		Yantis 1992; Yantis & Price 1993
1993							
1994							
1995							
1996							
1997							
1998							
1999							
2000							
2001							
2002							
2003							
2004							
2005							
2006							
2007	0 (19)	5 (11)	0 (17)	0 (5)	0 (3)	0 (11)	Forstner et al. 2007b
2008	0 (37)	0 (19)	5 (19)				Forstner et al. 2008

Values resulting from surveys are followed by number of sites surveyed in parentheses. * indicates vouchers exist (see Appendix B)

[a] Only south of Colorado River was surveyed this year in Bastrop County

[b] Number of sites surveyed is number of listening stops

Table 2 Numbers of individuals sampled from 2000 to 2008 per site by sex and geographic coordinates for each site

Site	Latitude	Longitude	Male	Female	Unknown	Total
Austin County						
AUS01p	29.87246	-96.36386	1			1
AUS02s	29.88395	-96.36161	1			1
AUS03p	29.87789	-96.35294	2			2
Bastrop Co. north						
BAN01p	30.16953	-97.24165	1			1
BAN02p	30.21626	-97.24172	79	13	16[a]	108
BAN03s	30.2106	-97.24802	1			1
BAN04p	30.20932	-97.24291	8		1[a]	9
BAN05p	30.21427	-97.23254	4	2	2[b]	8
BAN06p	30.21235	-97.23	12			12
BAN07p	30.2056	-97.23424	4			4
BAN08p	30.19918	-97.22197	13			13
BAN09p	30.1978	-97.21326	2	1		3
BAN10p	30.20198	-97.20898	4			4
BAN11p	30.17795	-97.2338	2			2
BAN12t	30.21586	-97.23886	3			3
BAN13t	30.21647	-97.24178	1	2		3
BAN14t	30.21658	-97.24097	1			1
BAN15t	30.21036	-97.23828	1			1
BAN16t	30.21436	-97.23325	2			2
BAN17t	30.21528	-97.23139	3			3
BAN18t	30.20008	-97.22266		2		2
BAN19t	30.2002	-97.22236	6	1		7
BAN20t	30.19989	-97.2172			1[a]	1
BAN21t	30.19981	-97.21703	1			1
BAN22t	30.19575	-97.21494		1		1
BAN23t	30.21586	-97.23928			1[a]	1
BAN24t	30.20953	-97.24197	1			1
BAN25t	30.20986	-97.24003	1			1
BAN26t	30.21029	-97.24548	2	2		4
BAN27s	30.30689	-97.16639			4[c]	4
BAN28p	30.24567	-97.22135	1			1
BAN29s	30.255	-97.22787	4			4
Bastrop Co. south						
BAS01p	30.13288	-97.26572	17		1[d]	18
BAS02p	30.14018	-97.2706	4			4
BAS03s	30.13874	-97.26881	2			2
BAS04p	30.14194	-97.26205	25	2	3[e]	30
BAS05s	30.13959	-97.26137	1			1
BAS06p	30.14236	-97.1958	17			17
BAS07p	30.0957	-97.23859	26			26
BAS08p	30.11438	-97.27673	10			10
BAS09p	30.09016	-97.23851	20			20
BAS10t	30.10428	-97.2682			1[a]	1
BAS11t	30.10094	-97.25169			3[a]	3
BAS12t	30.12065	-97.26009			2[a]	2
BAS13t	30.12069	-97.26204			1[a]	1
BAS14p	30.13941	-97.25118	3			3
BAS15p	30.13721	-97.24335	5			5

BAS16p	30.14108	-97.24349	1			1
BAS17p	30.12638	-97.23934	19			19
BAS18p	30.12633	-97.2337	8			8
Bastrop Co. GLR p12						
BAPp	30.19489	-97.24358	37	2		39
Colorado County						
COLs	29.84165	-96.4889	3			3
Lee County						
LEE01s	30.31281	-97.15247			1[d]	1
LEE02p	30.32482	-97.16896			6[b]	6
LEE03p	30.32764	-97.16957			4[b]	4
Leon County						
LEOp	31.0775	-96.19334	1			1
Milam County						
MILs	30.7135	-96.74612	3	1		4
Totals			363	29	47	439

Latitude and longitude in decimal degrees, WGS84 datum. Sites are grouped by county, and sites within Bastrop County are grouped into three subgroups (Bastrop Co. north, Bastrop Co. south, and GLR p12) based in part on general geographic proximity but also on results from analyses. The terminal letter in a site code represents the type of site: p = pond, s = site, and t = trap

[a] Juvenile

[b] Tadpole

[c] Sex not recorded

[d] Recorded as 'female?'

[e] Sex could not be determined

Table 3 Annealing T (ºC), WellRED fluorescent label, pooling group, number of alleles (*A*), and size range in bp for ten microsatellite loci in *Bufo houstonensis* (*n* = 439)

Locus	Annealing T	Label	Pooling	*A*	Range	Reference
BBR34-2	55	D4	singly	25	148-253	Simandle et al. 2006
BBR36	55	D4	singly	25	161-341	Simandle et al. 2006
BBR281	55	D4	singly	10	121-175	Simandle et al. 2006
BC52.03	55	D2	singly	11	387-439	Chan 2007
BC52.10	55	D4	1	17	127-227	Chan 2007
BC52.12	55	D4	singly	10	232-284	Chan 2007
bco15	55	D4	1	15	206-282	Chan 2007
BM224other	55	D2	2	12	58-82	Tikel et al. 2000
IHHH	60	D3	2	30	175-243	Gonzalez et al. 2004
IYY	55	D2	1	8	313-349	Gonzalez et al. 2004

Table 4 Individuals of other species used in phylogenetic analyses

Taxon	MF#	Locality	Sex	Haplotype	GenBank Accession No.
Bufo americanus (*n* = 8)					
	MF01103	NY: Otsego Co.	unknown	MF01103	HM021093
	MF02968	NY: Orange Co.	unknown	MF02968	HM021094
	MF07399	OK: Cleavand Co.	male	MF07399	HM021105
	MF08153	MO: Taney Co.	male	wooD	HM021107
	MF08154	MO: Taney Co.	male	MF08154	HM021108
	MF08155	MO: Taney Co.	male	wooD	
	MF08156	MO: Taney Co.	male	wooD	
	MF08157	MO: Taney Co.	male	wooD	
Bufo cognatus (*n* = 3)					
	MF03525	TX: Wichita Co.	male	MF03525	HM021095
	MF27040	TX: Randall Co.	juvenile	cogA	
	MF27054	TX: Parmer Co.	unknown	cogA	HM021118
Bufo fowleri (*n* = 3)					
	MF05186	GA: Carroll Co.	unknown	MF05186	HM021100
	MF10100	VA: Stafford Co.	female	fowA	HM021110
	MF10103	VA: Stafford Co.	female	fowA	
Bufo woodhousii (*n* = 20)					
	MF03523	TX: Wichita Co.	juvenile	wooC	
	MF05270	TX: Hill Co.	male	wooB	HM021101
	MF05271	TX: Hill Co.	unknown	wooB	
	MF05272	TX: Hill Co.	unknown	wooA	HM021102
	MF05273	TX: Hill Co.	unknown	wooB	
	MF05274	TX: Hill Co.	unknown	wooA	
	MF07398	OK: Cleavand Co.	male	MF07398	HM021104
	MF10031	TX: Hill Co.	female	wooA	
	MF20085	TX: Hill Co.	female	wooB	
	MF20086	TX: Hill Co.	female	wooB	
	MF20087	TX: Hill Co.	female	wooB	
	MF20088	TX: Hill Co.	female	wooA	
	MF20089	TX: Hill Co.	male	wooB	
	MF20945	TX: Hill Co.	male	wooB	
	MF20946	TX: Hill Co.	male	wooA	
	MF20947	TX: Hill Co.	male	wooB	
	MF20948	OK: Potowatomi Co.	female	wooC	HM021114
	MF21487	TX: Hill Co.	male	wooB	
	MF22054	TX: Aransas Co.	female	wooE	HM021115
	MF22055	TX: Aransas Co.	male	wooE	

Table 5 Comparison of genetic clustering analyses

Analysis	*n*	Spatial model used?	Description
GENELAND			
analysis A	439	yes	all individuals
analysis B	439	no	all individuals
missing data bias	72	yes	individuals with no missing data
oversampling bias	32 per subset	yes	10 subsets, see text
analysis C	427	yes	individuals from Bastrop and Lee counties
analysis D	427	no	individuals from Bastrop and Lee counties
analysis E	195, 154	yes	individuals assigned to cluster N or to cluster S, see text
analysis F	195, 154	no	individuals assigned to cluster N or to cluster S, see text
STRUCTURE			
analysis G	439	n/a	all individuals
missing data bias	72	n/a	individuals with no missing data
oversampling bias	32 per subset	n/a	10 subsets, see text
analysis H	427	n/a	individuals from Bastrop and Lee counties
analysis I	163, 135	n/a	individuals assigned to cluster N or to cluster S, see text

Table 6 Groups of sites constructed for analysis in BAYESASS version 1.3

BAYESASS Group	Site	GENELAND analysis A results	GENELAND analysis C results
Austin County			
Austin	AUS01p	U	—
	AUS02s	U	—
	AUS03p	U	—
Bastrop Co. north			
BAN09p	BAN09p	S	S_2
BAN27s	BAN27s	I+N+S+U	S_1+S_2
BANeast	BAN08p	N	N
	BAN10p	N	N
	BAN18t	N	N
	BAN19t	N	N
	BAN20t	N	N+S_2
	BAN21t	N	N+S_2
	BAN22t	S	N+S_1+S_2
BANnorth	BAN28p	N	N
	BAN29s	N	N
BANsouth	BAN01p	N	N
	BAN11p	S	S_1
BANwest	BAN02p	N	N
	BAN03s	N	N+S_2
	BAN04p	N	N
	BAN05p	N	N
	BAN06p	N	N
	BAN07p	N	N+X
	BAN12t	N	N
	BAN13t	N	N
	BAN14t	N	N
	BAN15t	N	N
	BAN16t	N	N
	BAN17t	N	N
	BAN23t	N	N
	BAN24t	N	N
	BAN25t	N	N
	BAN26t	N	N
Bastrop Co. south			
BAS06p	BAS06p	N	X
BAS08p	BAS08p	S	S_1+S_2
BAS15p	BAS15p	S	S_1+S_2
BAS18p	BAS18p	S	S_1+S_2
BASs1	BAS07p	S	S_1
	BAS09p	S	S_1
	BAS11t	S	S_1
	BAS16p	S	S_1
	BAS17p	S	S_1
BASs2	BAS01p	S	S_2
	BAS02p	S	S_2
	BAS03s	S	S_2
	BAS04p	S	S_2
	BAS05s	S	S_2
	BAS10t	S	S_2
	BAS12t	S	S_2

	BAS13t	S	S_2
	BAS14p	S	S_2
Bastrop Co. GLR p12			
BAPp	BAPp	N+S	X
Colorado County			
COLs	COLs	S	—
Lee County			
LEE01s	LEE01s	I+N+S+U	X
LEE02,03	LEE02p	S	S_2
	LEE03p	S	S_2
Leon County			
LEOp	LEOp	N	—
Milam County			
MILs	MILs	I	—

Groups were constructed based on geographic locality and assignments from GENELAND analyses

Table 7 Groups in BANwest (see Table 6) used for analysis in BAYESASS version 1.3

Site	GENELAND analysis A results	GENELAND analysis C results
BAN02p	N	N
BAN03s	N	$N+S_2$
BAN04p	N	N
BAN05p	N	N
BAN06p	N	N
BAN07p	N	N+X
BAN12t	N	N
BAN13t	N	N
BAN14t	N	N
BAN15t	N	N
BAN16t	N	N
BAN17t	N	N
BAN23t	N	N
BAN24t	N	N
BAN25t	N	N
BAN26t	N	N

Table 8 Numbers of individuals collected per site by year

Site	2000-01[a]	2002	2003	2004	2005	2006	2007-08[b]	Total
Austin County								
AUS01p							1	1
AUS02s							1	1
AUS03p							2	2
Bastrop Co. north								
BAN01p			1					1
BAN02p	15	46	4	7	32	4		108
BAN03s				1				1
BAN04p	2		1	4	2			9
BAN05p		5	1	2				8
BAN06p		4		1	7			12
BAN07p			4					4
BAN08p	4			4	3		2	13
BAN09p	3							3
BAN10p	4							4
BAN11p							2	2
BAN12t		1	1	1				3
BAN13t			1	2				3
BAN14t		1						1
BAN15t	1							1
BAN16t		1		1				2
BAN17t		3						3
BAN18t	1			1				2
BAN19t	1	5		1				7
BAN20t			1					1
BAN21t			1					1
BAN22t		1						1
BAN23t			1					1
BAN24t			1					1
BAN25t		1						1
BAN26t			3	1				4
BAN27s	4							4
BAN28p							1	1
BAN29s							4	4
Bastrop Co. south								
BAS01p						6	12	18
BAS02p							4	4
BAS03s							2	2
BAS04p							30	30
BAS05s							1	1
BAS06p			7		8		2	17
BAS07p						5	21	26
BAS08p							10	10
BAS09p						20		20
BAS10t					1			1
BAS11t					3			3
BAS12t					2			2
BAS13t					1			1
BAS14p							3	3
BAS15p						3	2	5
BAS16p						1		1

BAS17p						9	10	19
BAS18p						1	7	8
Bastrop Co. GLR p12								
BAPp			1		5	23	10	39
Colorado County								
COLs							3	3
Lee County								
LEE01s	1							1
LEE02p		6						6
LEE03p		4						4
Leon County								
LEOp						1		1
Milam County								
MILs							4	4
Total	36	78	28	26	64	73	134	439

[a] Only two individuals were collected in 2000; both are from BAN02p. All other individuals in 2000-01 were collected in 2001

[b] Only four individuals were collected in 2008; all four were collected from Austin County. All other individuals in 2007-08 were collected in 2007

Table 9 Comparison of support values in different phylogenetic analyses

Clade	MP[a]	ML[b]	Bayesian[c]
Occurring in Fig. 2			
clade Ib	100	100	100
clade Ia — clade Ib[d]	100	—	63
houB — houE — houF	100	100	99
clade Ic	100	100	76
houA — houC — houH — MF04876 hou — MF05707 hou[d]	—	—	73
clade Id	100	100	78
clade Ic — clade Id	100	100	92
clade I	100	100	89
fowA — MF05186 fow	100	100	100
clade IIIb	100	100	76
clade III[e]	100	100	77
Not occurring in Fig. 2			
clade Ia/Ib — clade Ie	100	—	—
clade I — MF08154 ame	100	—	—
clade I/MF08154 ame — clade III	100	—	—

[a] Bootstrap values from maximum parsimony analysis
[b] Bootstrap values from maximum likelihood analysis
[c] Posterior probabilities from Bayesian analysis
[d] Support values are not shown in Fig. 2
[e] All *B. woodhousii* occurred in this clade

Table 10 Summary of results from GENELAND version 3.1.4 analyses

		Counties							
			Bastrop						
	K	Austin	north	south	GLR p12	Colorado	Lee	Leon	Milam
*Dataset included all individuals (*n *= 439; analysis A)*									
	4	U	I+N+S+U	N+S	N+S	S	I+N+S+U	N	I
*Dataset included all individuals, without spatial (*n *= 439; analysis B)*									
	3	N+S+X	N+S+X	N+S+X	X	S	N+S+X	N	X
*Dataset included individuals for which there were no missing data (*n *= 72)*[a]									
	3	—	I+N	I+N+S	I+N	—	I	—	I
*Each dataset (*n *= 32) included 20 random individuals from Bastrop and Lee counties*									
1	4	U	N	S	N	I+S	—	N+S	I
2	4	U	N	N+S	N+S	S	—	N	I
3	4	U	I+N+S	I+S+U	—	S	I	N+S	I
4	4	U	I+N+S	I+S	I+S	I+S	N	I+S	I
5	4	U	N+S+U	N+S	N	I+S	—	S	I
6	4	U	N+S	S	S	S	—	N	I
7	5	U	N+S	S	S	S	—	E+N+S	I
8	6	U	N	S	N	S	E+I+O	O	I
9	4	U	S	S	S	S	—	I+N+S	I
10	5	U	E+N	S	N+S	S	E	E	I
*Dataset included only individuals from Bastrop and Lee counties (*n *= 427; analysis C)*									
	4	—	$N+S_1+S_2+X$	S_1+S_2	X	—	S_2+X	—	—
*Dataset included only individuals from Bastrop and Lee counties, without spatial (*n *= 427; analysis D)*									
	3	—	N+S+X	N+S+X		—	N+S+X	—	—
Second-order analyses ('N' n *= 195, 'S'* n *= 154; analysis E)*									
'N'	1	—	N	—	—	—	—	—	—
'S'	2	—	—	S_1+S_2	—	—	—	—	—
Second-order analyses, without spatial ('N' n *= 195, 'S'* n *= 154; analysis F)*									
'N'	1	—	N	—	—	—	—	—	—
'S'	2	—	—	S_1+S_2	—	—	—	—	—

All five counties and three groups within Bastrop County are shown (see Table 11 for assignments for sites within counties and Bastrop Co. groups). Clusters were designated E, I, N, O, S, S_1, S_2, U, and X. — indicates dataset included no individuals from that county or site

[a] Samples sizes for each group: north Bastrop (*n* = 52), south Bastrop (*n* = 15), and GLR p12 (*n* = 1), Lee (*n* = 3), and Milam (*n* = 1)

Table 11 Summary of GENELAND version 3.1.4 results per site by analysis

Site	*n*	GENELAND analysis					
		A	B	C	D	E	F
Austin County							
AUS01p	1	U	X	—	—	—	—
AUS02s	1	U	N+S+X	—	—	—	—
AUS03p	2	U	N+X	—	—	—	—
Bastrop Co. north							
BAN01p	1	N	N	N	N	N	N
BAN02p	108	N	N	N	N	N	N
BAN03s	1	N	N	N+S_2	N	N	N
BAN04p	9	N	N	N	N	N	N
BAN05p	8	N	N	N	N	N	N
BAN06p	12	N	N	N	N	N	N
BAN07p	4	N	N	N+X	N	N	N
BAN08p	13	N	N	N	N	N	N
BAN09p	3	S	S	S_2	S	—	—
BAN10p	4	N	N	N	N	N	N
BAN11p	2	S	S	S_1	S	—	—
BAN12t	3	N	N	N	N	N	N
BAN13t	3	N	N	N	N	N	N
BAN14t	1	N	N	N	N	N	N
BAN15t	1	N	N	N	N	N	N
BAN16t	2	N	N	N	N	N	N
BAN17t	3	N	N	N	N	N	N
BAN18t	2	N	N	N	N	N	N
BAN19t	7	N	N	N	N	N	N
BAN20t	1	N	N	N+S_2	N+S+X	N	N
BAN21t	1	N	N+S+X	N+S_2	N	N	N
BAN22t	1	S	N	N+S_1+S_2	N	—	—
BAN23t	1	N	N	N	N	N	N
BAN24t	1	N	N	N	N	N	N
BAN25t	1	N	N	N	N	N	N
BAN26t	4	N	N	N	N	N	N
BAN27s	4	I+N+S+U	N+S+X	S_1+S_2	S	—	—
BAN28p	1	N	N	N	N	—	—
BAN29s	4	N	N	N	N	N	N
Bastrop Co. south							
BAS01p	18	S	S	S_2	S	S_2	S_2
BAS02p	4	S	S	S_2	S	S_2	S_2
BAS03s	2	S	S	S_2	S	S_2	S_2
BAS04p	30	S	S	S_2	S	S_2	S_2
BAS05s	1	S	S	S_2	S	S_2	S_2
BAS06p	17	N	X	X	X	—	—
BAS07p	26	S	S	S_1	S	S_1	S_1
BAS08p	10	S	S	S_1+S_2	S	S_1	S_1+S_2
BAS09p	20	S	S	S_1	S	S_1	S_1
BAS10t	1	S	S	S_2	S	S_2	S_2
BAS11t	3	S	S	S_1	S	S_1	S_1
BAS12t	2	S	S	S_2	S	S_2	S_2
BAS13t	1	S	S	S_2	S	S_2	S_2
BAS14p	3	S	S	S_2	S	S_2	S_2

BAS15p	5	S	S	S_1+S_2	S	S_1+S_2	S_1
BAS16p	1	S	S	S_1	S	S_1	S_1
BAS17p	19	S	S	S_1	S	S_1	S_1
BAS18p	8	S	S	S_1+S_2	S	S_1+S_2	S_2
Bastrop Co. GLR p12							
BAPp	39	N+S	X	X	X	—	—
Colorado County							
COLs	3	S	S	—	—	—	—
Lee County							
LEE01s	1	I+N+S+U	X	X	X	—	—
LEE02p	6	S	S	S_2	S	—	—
LEE03p	4	S	N+S+X	S_2	N+S	—	—
Leon County							
LEOp	1	N	N	—	—	—	—
Milam County							
MILs	4	I	X	—	—	—	—

Analyses: (A) dataset included all individuals (n = 439), with the spatial model, K = 4; (B) dataset included all individuals (n = 439), without the spatial model, K = 3; (C) dataset included only individuals from Bastrop and Lee counties (n = 427), with the spatial model, K = 4; (D) dataset included only individuals from Bastrop and Lee counties (n = 427), without the spatial model, K = 3; (E) second-order analyses (n_N = 195, n_S = 154), with the spatial model, K_N = 1 and K_S = 2; and (F) second-order analyses (n_N = 195, n_S = 154), without the spatial model, K_N = 1 and K_N = 2. In all instances of partial assignments to multiple clusters, all individuals from a site were assigned by GENELAND to the same clusters. — indicates dataset included no individuals from that site

Table 12 Summary of results from STRUCTURE version 2.1 analyses

		Counties							
			Bastrop						
	K	Austin	north	south	GLR p12	Colorado	Lee	Leon	Milam
*Dataset included all individuals (*n = *439; analysis G)*									
	2	N	N+S	N+S	N+S	N+S	N+S	N+S	N+S
*Dataset included individuals for which there were no missing data (*n = *72)*[a]									
	3	—	I+N+S	I+N+S	I+S	—	I+S	—	I+S
*Each dataset (*n = *32) included 20 random individuals from Bastrop and Lee counties*									
1	3	U	N+S+U	N+S	N+S+U	N+S	—	N+S	N+S
2	5	U	$N+S_1+S_2+U$	$N+S_1+S_2$	$N+S_1+S_2+U$	$I+N+S_1+S_2$	—	$N+S_1+S_2$	I
3	4	U	I+N+S+U	I+N+S	—	N+S	I+N	I+N	I
4	4	U	I+N+S+U	I+N+S	I+N+S	I+N+S	N+S	N+S	I
5	4	U	I+N+S+U	I+N+S	I+N+S	I+N+S	—	I+N+S	I+N+S
6	7	U	$I+N_1+N_2+N_3+S_1+S_2+U$	$I+N_1+N_2+N_3+S_1+S_2$	$I+N_1+N_2+N_3+S_1+S_2$	$I+N_1+N_2+N_3+S_1+S_2$	—	$I+N_1+N_2+N_3+S_1+S_2$	$I+N_1+N_2+N_3$
7	4	U	I+N+S	I+N+S	I+N+S	I+N+S	—	I+N+S	I+N+S
8	2	N	N+S	N+S	N+S	N+S	S	S	S
9	2	N	N+S	S	S	S	—	S	S
10	2	N	N+S	N+S	N+S	S	S	S	S
*Dataset included only individuals from Bastrop and Lee counties (*n = *427; analysis H)*									
	2	—	N+S	N+S	N+S	—	N+S	—	—
Second-order analyses ('N' n = *163, 'S'* n = *135; analysis I)*									
'N'	2	—	N_1+N_2	—	—	—	—	—	—
'S'	2	—	—	S_1+S_2	—	—	—	—	—

All five counties and three groups within Bastrop County are shown (see Table 13 for assignments for sites within counties and Bastrop Co. groups). Clusters were designated I, N, N_1, N_2, N_3, S, S_1, S_2, and U. — indicates dataset included no individuals from that county or site

[a] Samples sizes for each group: north Bastrop (*n* = 52), south Bastrop (*n* = 15), and GLR p12 (*n* = 1), Lee (*n* = 3), and Milam (*n* = 1)

Table 13 Summary of STRUCTURE version 2.1 results per site by analysis

Site	*n*	STRUCTURE analysis		
		G	H	I
Austin County				
AUS01p	1	N	—	—
AUS02s	1	N	—	—
AUS03p	2	N	—	—
Bastrop Co. north				
BAN01p	1	N	N	N_1+N_2[a]
BAN02p	108	N, S, N+S (92, 3, 13)	N, S, N+S (95, 3, 10)	N_1, N_2, N_1+N_2 (24, 42, 26)[b]
BAN03s	1	N+S[a]	N+S[a]	—
BAN04p	9	N, N+S (8, 1)	N, N+S (8, 1)	N_1, N_2, N_1+N_2 (4, 1, 3)[b]
BAN05p	8	N, S (6, 2)	N, S, N+S (6, 1, 1)	N_1, N_2, N_1+N_2 (2, 3, 1)[b]
BAN06p	12	N, N+S (11, 1)	N, N+S (10, 2)	N_1, N_2, N_1+N_2 (7, 2, 2)[b]
BAN07p	4	N, N+S (2, 2)	N, N+S (3, 1)	N_1, N_1+N_2 (1, 1)[b]
BAN08p	13	N, S, N+S (11, 1, 1)	N, S (11, 2)	N_1, N_1+N_2 (6, 5)[b]
BAN09p	3	S, N+S (2, 1)	S, N+S (2, 1)	—
BAN10p	4	N, S (3, 1)	N, S (3, 1)	N_1, N_2 (2, 1)[b]
BAN11p	2	S	S	—
BAN12t	3	N	N	N_1, N_2 (2, 1)
BAN13t	3	N, N+S (1, 2)	N, N+S (1, 2)	N_2 (1)[b]
BAN14t	1	N+S[a]	N+S[a]	—
BAN15t	1	N	N	N_1+N_2 (1)
BAN16t	2	N, N+S (1, 1)	N, N+S (1, 1)	N_2 (1)[b]
BAN17t	3	N	N	N_1
BAN18t	2	N	N	N_2, N_1+N_2 (1, 1)
BAN19t	7	N, S (6, 1)	N, S (6, 1)	N_1, N_2, N_1+N_2 (4, 1, 1)[b]
BAN20t	1	N	N	N_1+N_2 (1)
BAN21t	1	N+S[a]	N+S[a]	—
BAN22t	1	N	N	N_1
BAN23t	1	N	N	N_2
BAN24t	1	N	N	N_1
BAN25t	1	N	N	N_1
BAN26t	4	N, N+S (3, 1)	N, N+S (3, 1)	N_1, N_1+N_2 (2, 1)[b]
BAN27s	4	N, S (1, 3)	N, S (1, 3)	—
BAN28p	1	N	N	N_1+N_2[a]
BAN29s	4	N, N+S (3, 1)	N, N+S (3, 1)	N_1, N_2, N_1+N_2 (1, 1, 1)[b]
Bastrop Co. south				
BAS01p	18	N, S, N+S (3, 14, 1)	N, S, N+S (3, 14, 1)	S_1, S_2, S_1+S_2 (3, 7, 4)[b]
BAS02p	4	S	S	S_2, S_1+S_2 (3, 1)
BAS03s	2	S	S	S_2
BAS04p	30	S	S	S_1, S_2, S_1+S_2 (2, 18, 10)
BAS05s	1	S	S	S_2
BAS06p	17	N, S, N+S (10, 4, 3)	N, S, N+S (10, 4, 3)	—
BAS07p	26	S, N+S (23, 3)	S, N+S (23, 3)	S_1, S_1+S_2 (16, 7)[b]
BAS08p	10	S, N+S (7, 3)	S, N+S (7, 3)	S_1, S_2, S_1+S_2 (1, 2, 4)[b]
BAS09p	20	N, S, N+S (2, 15, 3)	N, S, N+S (1, 16, 3)	S_1, S_2, S_1+S_2 (9, 2, 5)[b]
BAS10t	1	S	S	S_1+S_2[a]
BAS11t	3	S	S	S_1
BAS12t	2	S	S	S_2
BAS13t	1	S	S	S_2
BAS14p	3	S	S	S_1, S_2, S_1+S_2 (1, 1, 1)
BAS15p	5	S, N+S (4, 1)	N, S (1, 4)	S_1, S_1+S_2 (1, 3)[b]

BAS16p	1	S	N+S[a]	—
BAS17p	19	S, N+S (18, 1)	S, N+S (18, 1)	S_1, S_2, S_1+S_2 (7, 2, 9)[b]
BAS18p	8	S, N+S (6, 2)	S, N+S (6, 2)	S_1, S_2, S_1+S_2 (1, 1, 4)[b]
Bastrop Co. GLR p12				
BAPp	39	N, S, N+S (14, 17, 8)	N, S, N+S (14, 16, 9)	—
Colorado County				
COLs	3	S, N+S (2, 1)	—	—
Lee County				
LEE01s	1	N+S[a]	N+S[a]	—
LEE02p	6	S, N+S (4, 2)	S, N+S (4, 2)	—
LEE03p	4	S, N+S (2, 2)	N, S, N+S (1, 2, 1)	—
Leon County				
LEOp	1	N+S[a]	—	—
Milam County				
MILs	4	S, N+S (3, 1)	—	—

Analyses: (G) dataset included all individuals (n = 439), K = 2; (H) dataset included only individuals from Bastrop and Lee counties (n = 427), K = 2; (I) second-order analyses (n_N = 163, n_S = 135), K_N = 2 and K_S = 2. In instances where individuals at a site were assigned to different clusters, the number of individuals for each assignment is in parentheses. — indicates dataset included no individuals from that site

[a] All individuals from this site were assigned partial membership to the same multiple clusters by STRUCTURE

[b] The sample size is smaller here than in the other analyses because individuals with partial memberships and individuals assigned membership in a different cluster under analysis G were excluded from this analysis

Table 14 Characteristics of genetic diversity in all *Bufo houstonensis* and in the clusters identified by STRUCTURE version 2.1. Sample size (n), number of alleles (A), number of private alleles (A_p), allelic richness (R), and expected (H_E) and observed (H_O) heterozygosities are provided

Locus	all individuals[a]	STRUCTURE[b]		
		N	S	All
BBR34-2				
n	275	97	109	206
A	25	15	18	23
A_p	0	5	8	13
R	23.493	13.226	15.605	17.362
H_E	0.864	0.858	0.876	
H_O	0.542*	0.485*	0.606*	
BBR36				
n	421	159	148	307
A	25	19	19	25
A_p	0	6	6	12
R	22.940	16.061	16.889	19.130
H_E	0.909	0.902	0.899	
H_O	0.613*	0.610*	0.709*	
BBR281				
n	431	162	148	310
A	10	8	8	10
A_p	0	2	2	4
R	8.401	5.255	5.917	5.947
H_E	0.198	0.230	0.179	
H_O	0.086*	0.105*	0.088*	
BC52.03				
n	239	118	55	173
A	11	8	8	10
A_p	0	2	2	4
R	10.494	6.631	7.879	8.045
H_E	0.784	0.765	0.660	
H_O	0.184*	0.254*	0.145*	
BC52.10				
n	438	167	148	315
A	17	13	15	17
A_p	0	2	4	6
R	14.812	11.956	12.409	12.640
H_E	0.888	0.861	0.871	
H_O	0.548*	0.443*	0.669*	
BC52.12				
n	181	106	50	156
A	10	7	8	9
A_p	0	1	2	3
R	10.000	5.520	8.000	7.617
H_E	0.748	0.655	0.729	
H_O	0.188*	0.208*	0.180*	
bco15				
n	437	167	148	315
A	15	11	14	14
A_p	0	0	3	3

R	13.117	9.726	11.199	10.436
H_E	0.865	0.825	0.856	
H_O	0.714*	0.665*	0.764*	
BM224other				
n	439	167	148	315
A	12	10	7	10
A_p	0	3	0	3
R	9.545	7.786	6.657	7.258
H_E	0.755	0.721	0.728	
H_O	0.597*	0.653*	0.622*	
IHHH				
n	438	167	148	315
A	31	21	20	26
A_p	0	6	5	11
R	26.768	16.338	17.340	19.158
H_E	0.856	0.798	0.886	
H_O	0.671*	0.653*	0.764*	
IYY				
n	436	166	148	314
A	8	5	5	7
A_p	0	2	2	4
R	7.075	4.424	4.521	4.815
H_E	0.651	0.678	0.564	
H_O	0.475*	0.530*	0.486*	
Total				
n	439	167	148	315
A	164	117	122	151
A_p	—	29	34	63
Mean H_E	0.752	0.729	0.725	
Mean H_O	0.462	0.460	0.503	

Observed heterozygosities followed by a * significantly deviated from HWE before sequential Bonferroni correction. Observed heterozygosities in bold significantly deviated from HWE after sequential Bonferroni correction

[a]Allelic richness for all *B. houstonensis* was based on a minimum sample size of 181 individuals

[b]Allelic richness for STRUCTURE clusters was based on a minimum sample size of 50 individuals

Table 15 Characteristics of genetic diversity in nine groups identified via multiple methods (genetic clustering analyses, genetic diversity analyses, and migration rates analyses). Sample size (*n*), number of alleles (*A*), number of private alleles (A_p), allelic richness[a] (*R*), and expected (H_E) and observed (H_O) heterozygosities are provided

Locus	BAPp	BAS06p[b]	COLs[b]	LEOp[b]	I	N	S_1	S_2	U[b]	All
BBR34-2										
n	19	9	3	0	4	108	47	60	4	254
A	10	4	4	0	4	17	14	15	5	24
A_p	1	1	0	0	0	4	2	2	0	10
R	3.041	—	—	—	2.857	3.223	3.320	3.130	—	3.297
H_E	0.697	0.676	0.867	NA	0.786	0.754	0.832	0.854	0.857	
H_O	0.256*	0.176*	0.667	NA	1.000	0.255*	0.408*	0.453*	0.750	
BBR36										
n	38	15	3	0	4	183	71	75	4	393
A	8	6	4	0	3	20	16	17	2	25
A_p	0	0	0	0	0	5	2	1	0	8
R	2.357	—	—	—	2.414	3.468	3.326	3.496	—	3.516
H_E	0.621	0.806	0.800	NA	0.679	0.912	0.873	0.909	0.429	
H_O	0.308*	0.471*	0.667	NA	1.000	0.566*	0.732*	0.680*	0.000	
BBR281										
n	39	17	3	0	4	189	71	75	4	402
A	2	3	1	0	2	10	7	6	1	10
A_p	0	0	0	0	0	2	0	0	0	2
R	1.101	—	—	—	1.500	1.444	1.386	1.437	—	1.402
H_E	0.051	0.269	NA	NA	0.250	0.272	0.187	0.212	NA	
H_O	0.000*	0.176*	NA	NA	0.250	0.102*	0.056*	0.080	NA	
BC52.03										
n	21	10	1	0	2	139	12	40	0	225
A	5	2	1	0	1	11	4	6	0	11
A_p	0	0	0	0	0	4	0	0	0	4
R	2.530	—	—	—	1.000	2.846	2.281	2.276	—	2.921
H_E	0.698	0.649	0.533	NA	0.571	0.801	0.299	0.671	NA	
H_O	0.051*	0.000*	0.000	NA	0.000	0.168*	0.028*	0.067*	NA	
BC52.10										
n	39	17	3	1	4	196	71	74	4	409
A	9	7	3	1	2	14	13	11	2	16
A_p	0	0	0	0	0	2	2	0	0	4
R	3.219	—	—	—	1.786	3.317	3.389	3.156	—	3.394
H_E	0.855	0.756	0.733	NA	0.429	0.873	0.889	0.839	0.250	
H_O	0.538*	0.353*	0.333	NA	0.000	0.464*	0.775*	0.600*	0.250	
BC52.12										
n	2	0	0	0	3	119	7	40	0	171
A	3	0	0	0	2	8	4	6	0	10
A_p	1	0	0	0	1	1	0	0	0	3
R	3.000	—	—	—	1.933	2.482	2.546	2.390	—	2.742
H_E	0.100	NA	NA	NA	0.714	0.725	0.185	0.684	NA	
H_O	0.026*	NA	NA	NA	**0.000***	0.117*	0.014*	0.107*	NA	
bco15										
n	39	17	3	1	4	195	71	75	4	409
A	11	6	2	2	5	11	10	12	2	15
A_p	2	0	0	0	1	0	0	1	0	4
R	3.357	—	—	—	3.214	3.086	3.150	3.220	—	3.253
H_E	0.883	0.761	0.533	1.000	0.857	0.822	0.838	0.853	0.821	
H_O	0.692*	0.647	0.667	1.000	1.000	0.653*	0.746	0.773*	1.000	
BM224other										
n	39	17	3	1	4	196	71	75	4	410
A	6	6	3	2	3	11	7	6	3	11
A_p	0	0	0	0	0	3	0	0	0	3
R	2.376	—	—	—	2.557	2.694	2.595	2.818	—	2.787
H_E	0.629	0.725	0.733	1.000	0.714	0.727	0.684	0.759	0.679	
H_O	0.436*	0.471*	1.000	1.000	0.750	0.638*	0.535*	0.653*	0.750	
IHHH										
n	39	17	3	1	4	195	71	75	4	409
A	14	4	3	1	4	23	18	15	3	29
A_p	1	0	0	1	2	5	1	0	0	10

R	3.260	—	—	—	2.771	3.077	3.440	3.377	—	3.288
H_E	0.857	0.513	0.600	NA	0.750	0.811	0.895	0.884	0.607	
H_O	0.692*	0.235*	0.667	NA	0.500	0.668*	0.676*	0.800*	0.500	
IYY										
n	39	17	3	1	4	194	71	75	4	408
A	3	3	2	1	1	7	4	5	1	8
A_p	0	0	0	0	0	3	0	0	0	3
R	1.989	—	—	—	1.000	2.465	2.185	2.199	—	2.374
H_E	0.495	0.642	0.600	NA	NA	0.685	0.577	0.585	NA	
H_O	0.308*	0.294*	0.333	NA	NA	0.515*	0.465*	0.560*	NA	
Total										
n	39	17	3	1	4	196	71	75	4	410
A	71	41	23	7	27	132	97	99	19	159
A_p	5	1	0	1	4	29	7	4	0	51
Mean H_E	0.588	0.644	0.675	1.000	0.639	0.738	0.626	0.725	0.607	
Mean H_O	0.331	0.314	0.542	1.000	0.500	0.415	0.444	0.477	0.547	

Observed heterozygosities followed by a * significantly deviated from HWE before sequential Bonferroni correction. Observed heterozygosities in bold significantly deviated from HWE after sequential Bonferroni correction

[a] Allelic richness was based on a minimum sample size of 2 individuals

[b] *R* could not be calculated for this cluster because multiple loci had no genotyped individuals

Table 16 Pairwise F_{ST} values for four groups identified by GENELAND version 3.1.4 analysis A

Group	I ($n = 4$)	N ($n = 214$)	S ($n = 173$)	U ($n = 4$)
I	—			
N	**0.149**	—		
S	**0.109**	**0.035**	—	
U	**0.422**	**0.193**	**0.225**	—

Significant F_{ST} values are shown in bold

Table 17 Pairwise F_{ST} values for nine groups detected via multiple methods

Group	BAPp (n = 39)	BAS06p (n = 17)	COLs (n = 3)	I (n = 4)	LEOp (n = 1)	N (n = 196)	S_1 (n = 71)	S_2 (n = 75)	U (n = 4)
BAPp	—								
BAS06p	**0.099**	—							
COLs	**0.117**	**0.118**	—						
I	**0.195**	**0.253**	**0.182**	—					
LEOp	0.275	0.336	0.400	0.383	—				
N	**0.081**	**0.080**	**0.094**	**0.143**	**0.204**	—			
S_1	**0.091**	**0.118**	**0.077**	**0.171**	**0.214**	**0.081**	—		
S_2	**0.082**	**0.106**	0.051	**0.119**	**0.215**	**0.046**	**0.051**	—	
U	**0.268**	**0.285**	**0.339**	**0.400**	0.565	**0.196**	**0.199**	**0.223**	—

Significant F_{ST} values are shown in bold

Table 18 Summary of results from Mantel tests, as calculated in AIS version 1.0. For each dataset, regressions were performed on geographic distances and on log transformed geographic distances. Number of samples (*n*), correlation coefficient (*r*), and significance value (*P*) are provided

Analysis	*r*	*P*
*Microsatellites, all individuals (*n = *439)*		
geographic distance (km)	0.0698	<0.01
log transformed geographic distance	0.1186	<0.0001
*Microsatellites, only Bastrop and Lee counties (*n = *427)*		
geographic distance (km)	0.1411	<0.0001
log transformed geographic distance	0.1177	<0.0001
*Microsatellites, only Bastrop County (*n = *416)*		
geographic distance (km)	0.1039	<0.0001
log transformed geographic distance	0.0973	<0.0001
*mtDNA, all individuals (*n = *107)*		
geographic distance (km)	0.1591	<0.005
log transformed geographic distance	0.1488	<0.0001
*mtDNA, only Bastrop County (*n = *95)*		
geographic distance (km)	0.0938	<0.01
log transformed geographic distance	0.0631	<0.01

Table 19 Migration rates among *Bufo houstonensis* groups described in Table 6, obtained using BAYESASS version 1.3

	INTO							
	Austin	Bastrop Co. north						
FROM		BAN09p	BAN27s	BANeast	BANnorth	BANsouth	BANwest	BAS06p
Austin	**0.943**	0.012	0.011	0.002	0.011	0.011	0.001	0.001
BAN09p	0.003	**0.733**[a]	0.011	0.003	0.011	0.011	0.029	0.001
BAN27s	0.004	0.012	**0.722**	0.003	0.011	0.012	0.001	0.001
BANeast	0.003	0.012	0.023	**0.784**	0.054	0.012	0.070	0.001
BANnorth	0.003	0.012	0.011	0.003	**0.713**	0.012	0.001	0.001
BANsouth	0.003	0.012	0.011	0.003	0.010	**0.733**[a]	0.001	0.001
BANwest	0.003	0.034	0.015	**0.152**	0.056	0.022	**0.890**	0.001
BAS06p	0.003	0.011	0.012	0.003	0.013	0.012	0.001	**0.982**
BAS08p	0.004	0.013	0.011	0.003	0.011	0.011	0.001	0.001
BAS15p	0.003	0.012	0.011	0.002	0.010	0.014	0.001	0.001
BAS18p	0.003	0.011	0.011	0.002	0.012	0.012	0.001	0.001
BASs1	0.003	0.016	0.037	0.014	0.010	0.040	0.001	0.001
BASs2	0.004	0.040	0.011	0.004	0.011	0.013	0.001	0.001
BAPp	0.003	0.012	0.013	0.009	0.011	0.026	0.001	0.001
COLs	0.003	0.011	0.010	0.003	0.011	0.011	0.001	0.001
LEE01s	0.003	0.011	0.012	0.003	0.011	0.011	0.001	0.001
LEE02,03	0.003	0.012	0.046	0.003	0.011	0.011	0.001	0.001
LEOp	0.003	0.012	0.012	0.003	0.011	0.012	0.001	0.001
MILs	0.003	0.012	0.010	0.003	0.012	0.013	0.001	0.001

Table 19 continued

	INTO										
	Bastrop Co. south					BAPp	COLs	LEE01s	LEE02,03	LEOp	MILs
FROM	BAS08p	BAS15p	BAS18p	BASs1	BASs2						
Austin	0.008	0.011	0.009	0.000	0.001	0.002	0.012	0.012	0.002	0.012	0.012
BAN09p	0.009	0.010	0.009	0.000	0.001	0.001	0.012	0.010	0.002	0.013	0.012
BAN27s	0.008	0.010	0.009	0.000	0.001	0.002	0.010	0.012	0.002	0.012	0.013
BANeast	0.008	0.013	0.009	0.000	0.001	0.005	0.013	0.013	0.002	0.011	0.014
BANnorth	0.008	0.011	0.008	0.000	0.001	0.002	0.012	0.011	0.002	0.011	0.011
BANsouth	0.008	0.012	0.008	0.000	0.001	0.002	0.012	0.012	0.002	0.011	0.013
BANwest	0.017	0.021	0.018	0.000	0.002	0.029	0.017	0.012	0.002	0.016	0.018
BAS06p	0.008	0.037	0.010	0.000	0.001	0.003	0.013	0.017	0.002	0.012	0.012
BAS08p	**0.694**	0.010	0.008	0.000	0.001	0.002	0.011	0.011	0.002	0.011	0.013
BAS15p	0.008	**0.715**	0.010	0.000	0.001	0.002	0.012	0.011	0.002	0.013	0.012
BAS18p	0.009	0.010	**0.706**	0.000	0.001	0.002	0.012	0.012	0.002	0.012	0.029
BASs1	**0.108**[a]	0.056	0.083[a]	**0.994**	0.045	0.006	0.027	0.011	0.002	0.015	0.017
BASs2	0.052	0.014	0.045	0.000	**0.927**	0.002	0.036	0.012	0.002	0.014	0.012
BAPp	0.013	0.012	0.021	0.000	0.009	**0.934**	0.023	0.015	0.002	0.012	0.039
COLs	0.009	0.011	0.009	0.000	0.001	0.002	**0.731**[a]	0.011	0.002	0.013	0.011
LEE01s	0.009	0.012	0.008	0.000	0.001	0.002	0.011	**0.781**[a]	0.002	0.012	0.013
LEE02,03	0.010	0.012	0.012	0.000	0.001	0.001	0.012	**0.113**	**0.963**	0.015	0.013
LEOp	0.008	0.011	0.009	0.000	0.001	0.002	0.012	0.013	0.002	**0.772**[a]	0.013
MILs	0.008	0.011	0.010	0.000	0.001	0.001	0.012	0.012	0.002	0.012	**0.724**

Source groups (FROM) are listed in the left-hand column, receiving groups (INTO) are listed across the top row. Bold values are migration rates >0.1. Boxes frame values within subgroup Bastrop Co. north and within subgroup Bastrop Co. south. Sites were grouped based on geographic locality and resulting assignments from GENELAND analyses (see Table 6)

[a] Standard deviation was 0.052-0.081. All other standard deviations were <0.05

Table 20 Migration rates among *Bufo houstonensis* groups in BANwest described in Table 7, obtained using BAYESASS version 1.3

	INTO							
	sites near BAN02p					sites near BAN04p		
FROM	BAN02p	BAN12t	BAN13t	BAN14t	BAN23t	BAN03s	BAN04p	BAN15t
BAN02p	**0.819**	0.022	0.056[b]	0.018	0.023	0.015	0.015[a]	0.016
BAN12t	0.001	**0.733**[b]	0.015	0.014	0.013	0.015	0.004	0.016
BAN13t	0.001	0.014	**0.733**[b]	0.014	0.014	0.014	0.003	0.013
BAN14t	0.001	0.015	0.015	**0.775**[b]	0.014	0.015	0.003	0.014
BAN23t	0.001	0.014	0.016	0.014	**0.777**[b]	0.013	0.004	0.015
BAN03s	0.001	0.014	0.014	0.016	0.014	**0.778**[b]	0.004	0.016
BAN04p	0.079	0.044	0.017	0.021	0.014	0.024	**0.936**[b]	0.023
BAN15t	0.001	0.014	0.015	0.014	0.015	0.015	0.004	**0.774**[b]
BAN24t	0.001	0.016	0.016	0.015	0.016	0.015	0.003	0.014
BAN25t	0.001	0.014	0.014	0.013	0.014	0.014	0.003	0.015
BAN26t	0.001	0.014	0.016	0.016	0.015	0.015	0.003	0.015
BAN05p	0.001	0.013	0.014	0.015	0.014	0.013	0.003	0.013
BAN06p	0.065	0.033	0.015	0.012	0.014	0.013	0.005	0.014
BAN16t	0.023	0.015	0.016	0.015	0.013	0.015	0.004	0.014
BAN17t	0.001	0.014	0.015	0.014	0.016	0.013	0.003	0.014
BAN07p	0.001	0.013	0.015	0.013	0.015	0.013	0.003	0.014

Table 20 continued

	INTO							
	sites near BAN04p			sites near BAN05p				BAN07p
FROM	BAN24t	BAN25t	BAN26t	BAN05p	BAN06p	BAN16t	BAN17t	
BAN02p	0.014	0.015	0.046	0.087[a]	0.033[a]	0.039	0.021	0.058
BAN12t	0.015	0.012	0.014	0.010	0.008	0.015	0.016	0.014
BAN13t	0.014	0.015	0.013	0.009	0.007	0.014	0.016	0.014
BAN14t	0.014	0.013	0.014	0.010	0.009	0.015	0.014	0.013
BAN23t	0.014	0.015	0.014	0.010	0.008	0.015	0.014	0.013
BAN03s	0.013	0.015	0.013	0.009	0.008	0.014	0.015	0.014
BAN04p	0.026	0.023	0.058	0.058	**0.167**[ab]	0.021	0.051	0.047
BAN15t	0.015	0.013	0.013	0.010	0.009	0.013	0.015	0.013
BAN24t	**0.776**[b]	0.014	0.014	0.010	0.008	0.013	0.015	0.012
BAN25t	0.013	**0.781**[b]	0.013	0.009	0.008	0.014	0.015	0.014
BAN26t	0.015	0.014	**0.720**	0.010	0.008	0.017	0.015	0.013
BAN05p	0.013	0.013	0.014	**0.702**	0.008	0.015	0.015	0.014
BAN06p	0.015	0.015	0.013	0.039	**0.694**	0.015	0.016	0.014
BAN16t	0.014	0.014	0.014	0.010	0.008	**0.749**[b]	0.015	0.014
BAN17t	0.016	0.015	0.013	0.009	0.008	0.015	**0.733**[b]	0.013
BAN07p	0.013	0.014	0.014	0.009	0.008	0.015	0.014	**0.721**

Source groups (FROM) are listed in the left-hand column, receiving groups (INTO) are listed across the top row. Bold values are migration rates >0.1

[a] For these values, the other nine runs did not converge on a similar solution

[b] Standard deviation was 0.051-0.080. All other standard deviations were <0.05

Table 21 Analysis of molecular variance (AMOVA) results, using microsatellite data (A-H) or sequence data (I-J), for different hierarchical models. Genetic variance is partitioned among (A) groups identified by STRUCTURE version 2.1; (B) and (D) groups identified by GENELAND version 3.1.4; (C) and (E) groups detected via multiple methods; (F) and (G) geographic groups; (H) sites then years; (I) sites using mtDNA; or (J) four groups in Bastrop County, using mtDNA

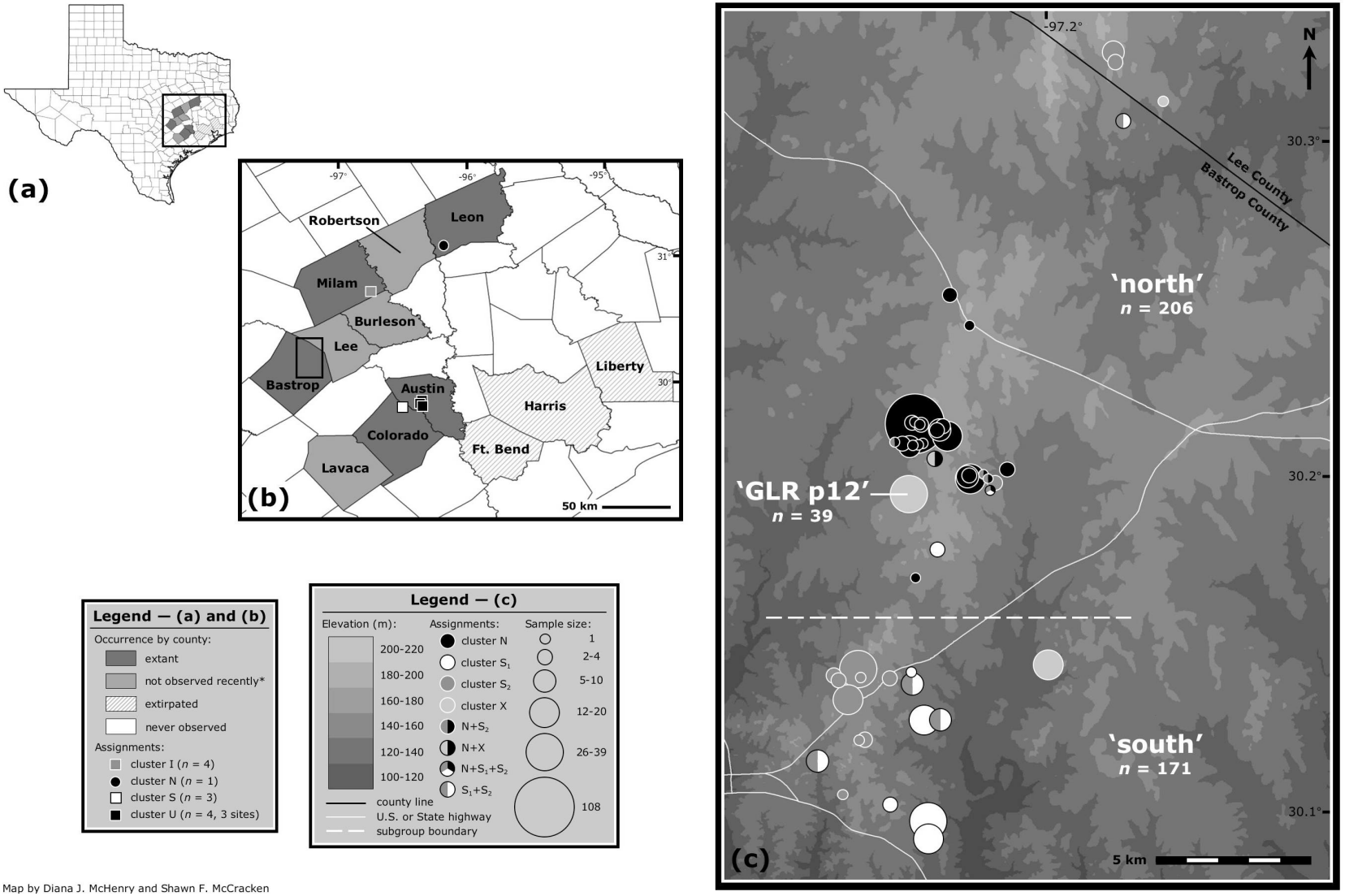
Hierarchical models	Source of variation	% total variance	*P*
(A) *Groups identified by STRUCTURE analysis G (N and S)*			
	Among groups	3.48	<0.00001
	Among sites	7.31	<0.00001
	Within sites	89.21	<0.00001
(B) *Groups identified by GENELAND analysis A (I, N, S, and U)*			
	Among groups	4.01	<0.00001
	Among sites	3.82	<0.00001
	Within sites	92.17	<0.00001
(C) *Six groups detected via multiple methods (BAPp, BAS06p, I, N, S, and U)*			
	Among groups	4.80	<0.00001
	Among sites	3.44	<0.00001
	Within sites	91.76	<0.00001
(D) *Groups identified across GENELAND analyses A, C, and E (I, N, S_1, S_2, and U)*			
	Among groups	3.81	<0.00001
	Among sites	3.52	<0.00001
	Within sites	92.67	<0.00001
(E) *Nine groups detected via multiple methods (BAPp, BAS06p, COLs, I, LEOp, N, S_1, S_2, and U)*			
	Among groups	4.71	<0.00001
	Among sites	3.10	<0.01
	Within sites	92.19	<0.00001
(F) *Two geographic groups (sites in Bastrop and Lee counties vs. sites in all others)*			
	Among groups	3.05	<0.01
	Among sites	6.80	<0.00001
	Within sites	90.15	<0.00001
(G) *Two geographic groups (sites in Austin County vs. sites in all others)*			
	Among groups	19.10	<0.00001
	Among sites	5.46	<0.00001
	Within sites	75.44	<0.00001
(H) *Sites then years*			
	Among sites	4.80	<0.00001
	Among years	3.36	<0.01
	Within years	91.84	<0.00001
*(I) Sites, using mtDNA (*n = *134)*			
	Among groups	6.34	ns
	Among sites	28.55	ns
	Within sites	65.12	<0.00001
(J) *Groups in Bastrop County, using mtDNA (BAPp, N, S_1, and S_2;* n = *104)*			
	Among groups	14.11	ns
	Among sites	16.30	<0.05
	Within sites	69.59	<0.00001

Table 22 Average uncorrected pairwise divergences (below diagonal, shaded) and estimated divergence dates (mya, along and above diagonal) after excluding shared haplotypes in *Bufo americanus* and *B. houstonensis*

	B. cognatus	*B. americanus*	*B. fowleri*	*B. houstonensis*	*B. woodhousii*
B. cognatus	0.114	4.806 – 4.410	5.549 – 5.101	4.749 – 4.638	4.754 – 4.753
B. americanus	15.368 %	0.856 – 0.171	2.123 – 1.994	1.314 – 0.171	1.142 – 0.229
B. fowleri	17.509 %	6.528 %	1.548	2.296 – 1.886	2.174 – 1.892
B. houstonensis	15.585 %	2.666 %	6.827 %	0.799 – 0.057	1.542 – 0.057
B. woodhousii	15.478 %	3.368 %	6.723 %	3.822 %	0.572 – 0.057

mya = million years ago

Fig. 1 (a) Occurrence of *Bufo houstonensis* in the state of Texas by county. Inset is Fig. 1(b). **(b)** Sites sampled outside of Bastrop and Lee counties; symbols show population assignments from GENELAND version 3.1.4 analysis of all individuals (analysis A). Inset is Fig. 1(c). **(c)** Sites sampled in Bastrop and Lee counties; symbols show population assignments from GENELAND version 3.1.4 analysis of only Bastrop and Lee counties (analysis C) and sample sizes. The three geographic subgroups within Bastrop County (north, south, and GLR p12; see Table 2; white dashed line is the approximate boundary between subgroups north and south) and their sample sizes are also indicated



Map by Diana J. McHenry and Shawn F. McCracken

Fig. 2 Bayesian consensus phylogram of 26 unique mtDNA haplotypes (194 individuals) rooted with *Bufo cognatus*. Haplotypes occurring in multiple individuals have four letter designations followed by sample size; haplotypes occurring in only one individual are denoted by MF# followed by an abbreviation of the specific epithet (e.g., cog = *B. cognatus*). MP bootstraps, ML bootstraps, and Bayesian posterior probabilities are shown above branches. Black vertical bars indicate the three clades (I, II, and III) involving *B. americanus*, *B. houstonensis*, and *B. woodhousii*. Hatched vertical bars indicate finer scale clades (see also Table 8). *B. houstonensis* occur in clades Ib, Ic, Id, IIIa (22 of 27), and IIIb (3 of 5 in wooC). *B. woodhousii* are found in clade III. *B. americanus* are shaded; all four individuals in haplotype wooD were *B. americanus*. *B. americanus* occurring in clades Ia and Ie were collected in New York, while those in clades II and IIIb were collected in Missouri and Oklahoma

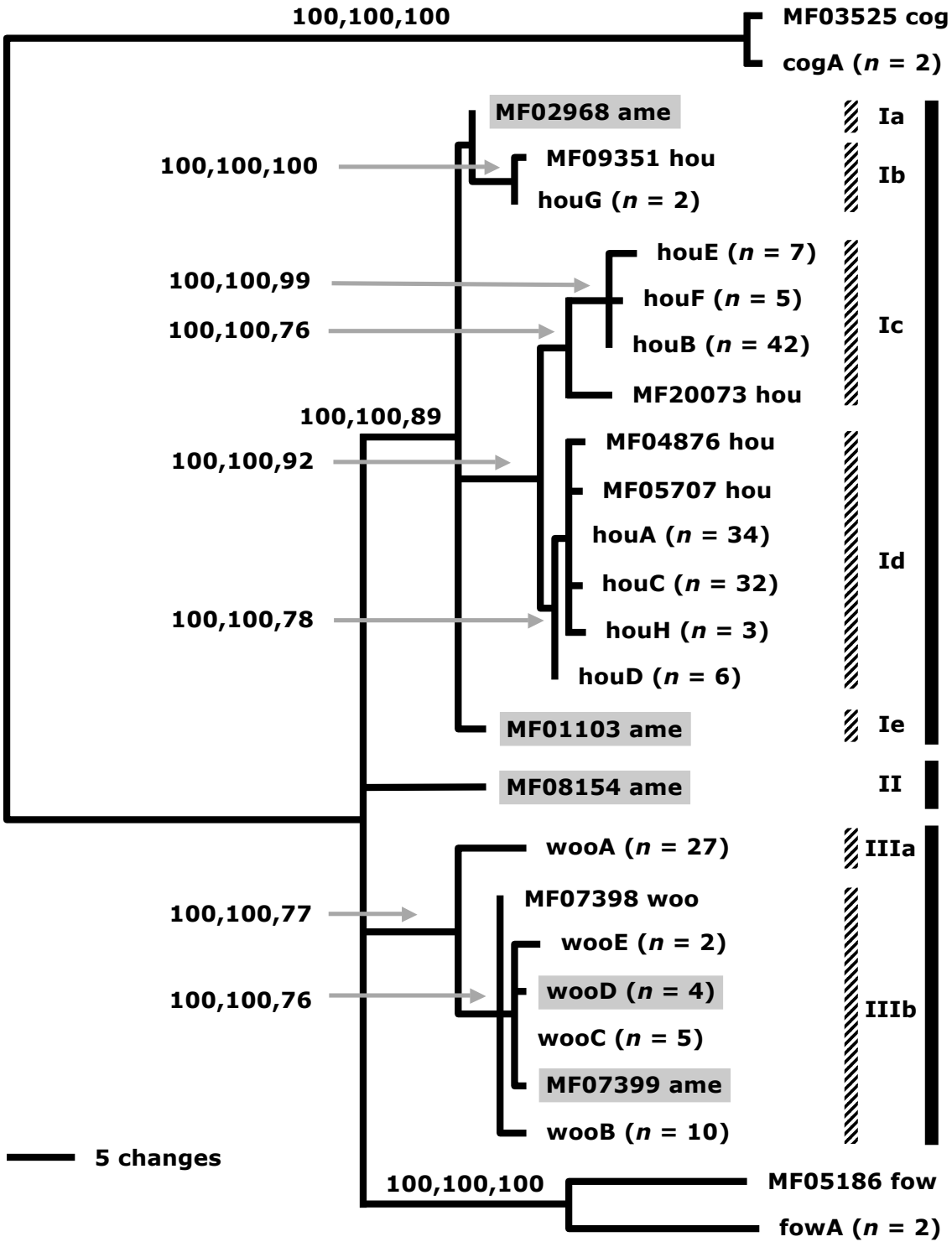
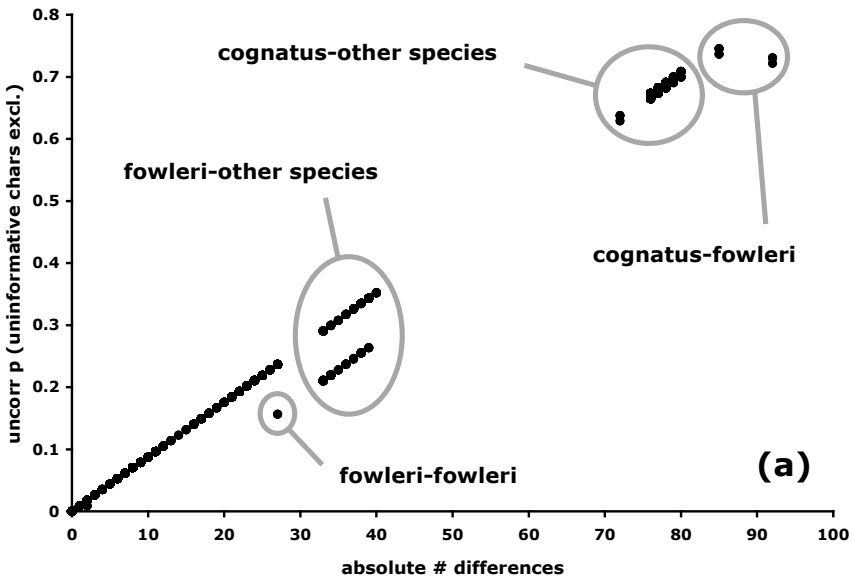

100,100,100
MF03525 cog
cogA (n = 2)
MF02968 ame
Ia
100,100,100
MF09351 hou
houG (n = 2)
Ib
houE (n = 7)
100,100,99
houF (n = 5)
100,100,76
houB (n = 42)
Ic
MF20073 hou
100,100,89
MF04876 hou
100,100,92
MF05707 hou
houA (n = 34)
houC (n = 32)
Id
100,100,78
houH (n = 3)
houD (n = 6)
MF01103 ame
Ie
MF08154 ame
II
wooA (n = 27)
IIIa
MF07398 woo
100,100,77
wooE (n = 2)
100,100,76
wooD (n = 4)
wooC (n = 5)
IIIb
MF07399 ame
wooB (n = 10)
5 changes
100,100,100
MF05186 fow
fowA (n = 2)

Fig. 3 (a) Uncorrected pairwise distance (after excluding uninformative characters) plotted against absolute number of differences among *Bufo americanus*, *B*. *cognatus*, *B*. *fowleri*, *B*. *houstonensis*, and *B*. *woodhousii*. Pairwise comparisons of fowleri-fowleri, fowleri-other species, cognatus-fowleri, and cognatus-other species are indicated by grey circles. Data points not enclosed in a grey circle are comparisons within cognatus and among or within americanus, houstonensis, and woodhousii. Saturation was observed at differences >80 (cognatus-fowleri comparisons). **(b)** Uncorrected pairwise distance of transitions (black squares) and transversions (grey circles) plotted against absolute distance. Saturation of transitions was observed at distances >0.125 (pairwise comparisons involving cognatus)


cognatus-other species
fowleri-other species
cognatus-fowleri
fowleri-fowleri
(a)
uncorr p (uninformative chars excl.)
absolute # differences
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80
90
100

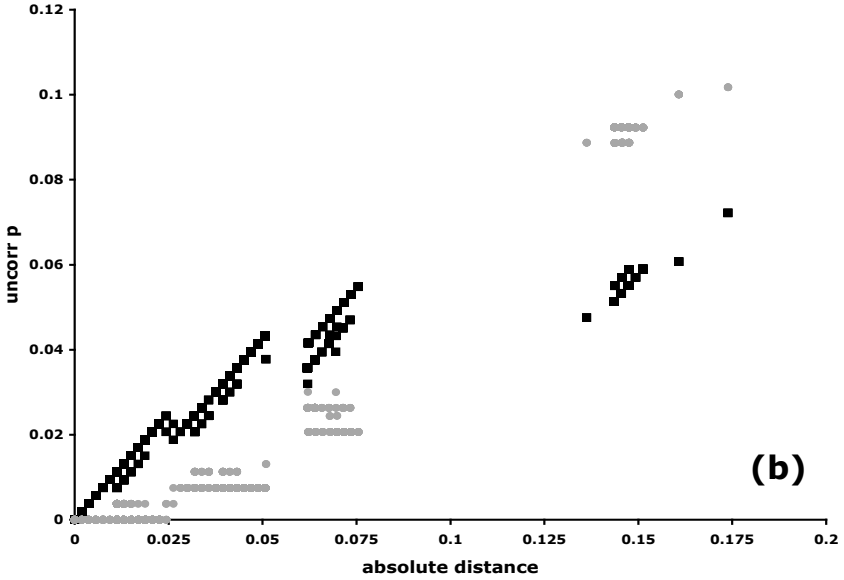
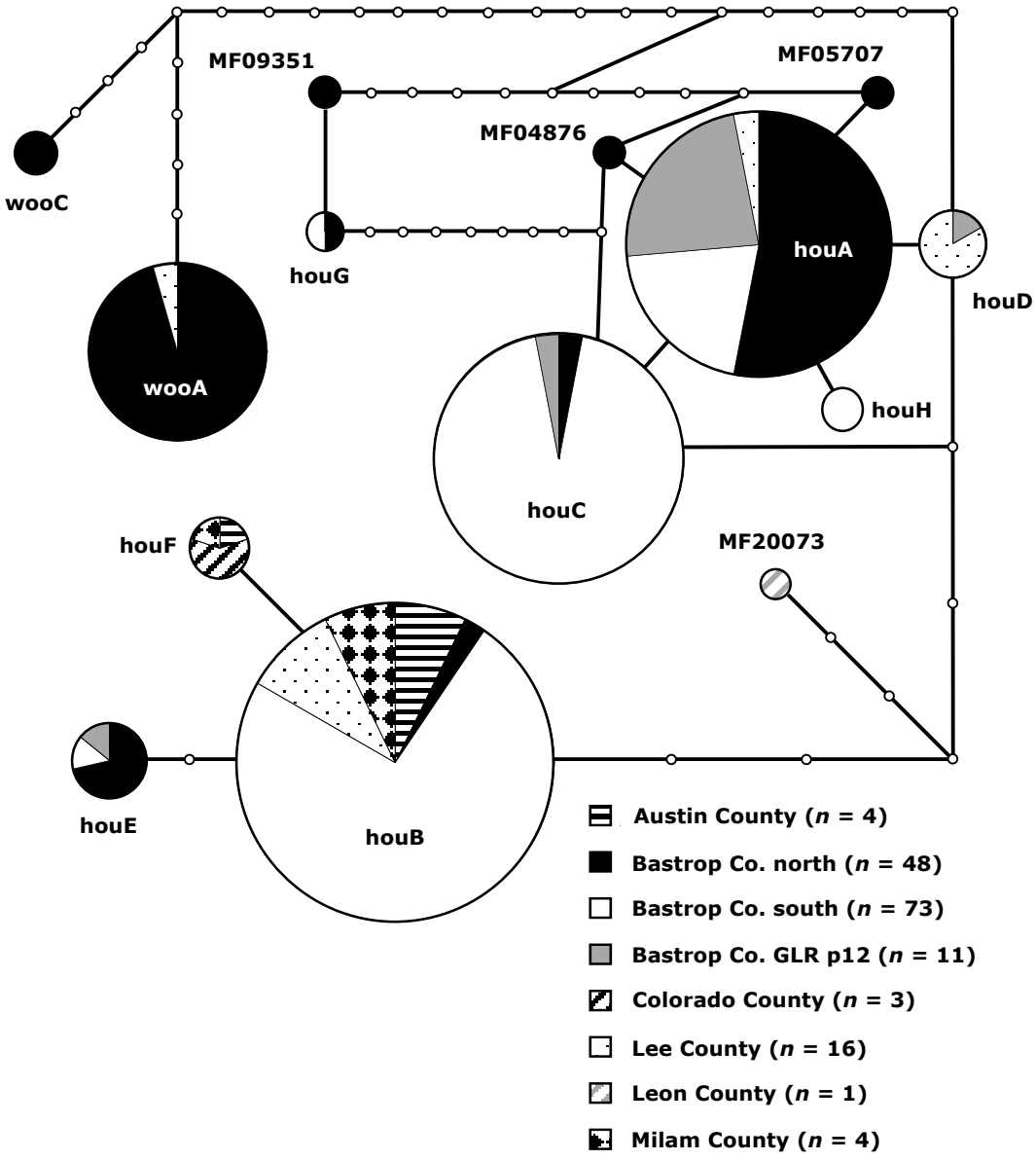

(b)
uncorr p
absolute distance
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.025
0.05
0.075
0.1
0.125
0.15
0.175
0.2

Fig. 4 Statistical parsimony network of 14 mtDNA haplotypes in 160 *Bufo houstonensis*. Circle size is proportional to number of individuals: houA (*n* = 34), houB (*n* = 42), houC (*n* = 32), houD (*n* = 6), houE (*n* = 7), houF (*n* = 5), houG (*n* = 2), houH (*n* = 3), MF04876 (*n* = 1), MF05707 (*n* = 1), MF09351 (*n* = 1), MF20073 (*n* = 1), wooA (*n* = 22), and wooC (*n* = 3). Each line represents a single mutation; small empty circles represent nonsampled or extinct haplotypes. Shading indicates geographic groups


MF09351
MF05707
MF04876
wooC
houG
houA
houD
wooA
houH
houC
houF
MF20073
houE
houB
Austin County (n = 4)
Bastrop Co. north (n = 48)
Bastrop Co. south (n = 73)
Bastrop Co. GLR p12 (n = 11)
Colorado County (n = 3)
Lee County (n = 16)
Leon County (n = 1)
Milam County (n = 4)

Chapter 3

GENETIC VARIATION AND POPULATION STRUCTURE IN THE COASTAL PLAIN TOAD (*BUFO NEBULIFER*)

ABSTRACT.—The coastal plain toad, *Bufo nebulifer*, is sympatric with the endangered Houston toad, *Bufo houstonensis*, through all of the latter species' range. Examination of more common sympatric congeners may be necessary to effectively manage rare or endangered species, especially in cases where widespread or frequent hybridization is known or when human activities increase the rates of hybridization. *Bufo houstonensis* and *B. nebulifer* are known to hybridize, and while recent work has been conducted to investigate the genetic diversity and structure within *B. houstonensis*, no comparable data yet exist for *B. nebulifer*. Here I investigate population genetic structure and diversity, including migration/movement rates, at both the landscape and fine scales. Much of the range was sampled and nine groups were recovered. Their relationships may be explained by a long residence in much of its present-day distribution (at least tens of thousands of years), with a history of range contraction during glaciation and re-expansion following the retreat of glaciers. *Bufo houstonensis* and *B. nebulifer* have comparable levels of genetic diversity, but *B. nebulifer* seems to migrate less frequently or over less distance than its endangered congener.

For endangered or rare taxa that occur in sympatry with common and/or abundant congeners, hybridization can be a threat to the survival of the species via gamete wastage, population-wide lowered fitness due to presence of less fit hybrids, and extinction through introgression or through competition with heterotic hybrids (Levin et al. 1996; Allendorf et al. 2001). Accordingly, in addition to understanding the biology of a rare species, we must also investigate its sympatric congeners. This is especially important in taxa with widespread or frequent hybridization or when human activities may be increasing the rates of hybridization (e.g., introduction of nonnatives or habitat alteration). Hybridization among toad species in the family Bufonidae is well-known and widespread (Blair 1959; 1963; Brown 1971; Blair 1972; Hillis et al. 1984; Gergus et al. 1999; Vogel and Johnson 2008); hence, for the endangered Houston toad, *Bufo houstonensis*, (= *Anaxyrus houstonensis*, Frost et al. 2006b), examination of its abundant sympatric relatives is essential to its recovery effort.

The coastal plain toad, *Bufo nebulifer* (= *Incilius nebulifer*, Mulcahy and Mendelson 2000; Frost et al. 2006b; Frost et al. 2006a; Frost et al. 2009), is a common and abundant toad throughout its range (from Veracruz, Mexico into northern Texas and from the Big Bend area in Texas east to Louisiana, Fig. 1a; Hammerson and Canseco-Márquez 2004; IUCN 2009) occurring throughout the entire range of *B. houstonensis*, sometimes chorusing at the same pond at the same time (Brown 1971; Hillis et al. 1984; Price 1990; Forstner 2002). Many basic aspects of the biology of *B. nebulifer* have been investigated: mating call and sexual selection characteristics (McAlister 1961; Porter 1964; Wagner and Sullivan 1995), parotoid gland vasculature (Hutchinson and Savitsky

2004), temperature tolerance (Hubbs et al. 1963), orientation and homing behavior (Awbrey 1963; Grubb 1970; 1973a; b), growth and dispersal in juveniles (Blair 1953), environmental conditions of a breeding pond (Blair 1960), and hybridization with other species (Thornton 1955; Blair 1959; Kennedy 1961; Blair 1972; Hillis et al. 1984; Vogel and Johnson 2008). Recently, molecular methods have been employed to reconstruct the phylogenetic relationship of *B. nebulifer* to other bufonids and to detect cryptic hybrids (Pauly et al. 2004; Frost et al. 2006b; Vogel and Johnson 2008).

Here I use molecular methods to examine, for the first time, population genetic structure within *B. nebulifer*. Besides expanding knowledge of this common and successful species, understanding its genetic diversity and structure may provide insight into why *B. houstonensis* is rare and how that endangered taxon may be more effectively managed. To truly appreciate the diversity and structure within a rare species, it should be placed in the context of its relatives (Karron 1987; Gitzendanner and Soltis 2000), particularly those in sympatry; for example, heterozygosity is one way to describe levels of variation and is much more valuable when a comparator, say heterozygosity in another congener, is available. Barriers to gene flow can be detected using population genetic approaches. With taxa where human-altered habitats increase the opportunity for hybridization events, understanding these barriers in both species may improve conservation management strategies. Similarly, genetic structure and diversity may now, because molecular methods are efficient enough to have come into more widespread use, be used in conjunction with other known aspects of the life histories of rare and common species. For example, clutch size and breeding season length differ in *B. houstonensis* and

B. *nebulifer* (Mendelson 2005; Shepard and Brown 2005); these differences might have an effect on population genetic characteristics. Coupling genetic diversity and structure of both species with what has already been determined about their life histories may reveal why some species are common and others are rare. In my study, I address the following questions using mitochondrial sequence data and nuclear microsatellite loci for samples collected over much of the range: (1) what is a population in *B*. *nebulifer* and how many exist? (2) what are the levels of genetic diversity within and among populations? (3) how differentiated are populations? and (4) what are the patterns of movement, as reconstructed from genetic connectivity, at the landscape- and fine-scale levels?

MATERIALS AND METHODS

Sampling.—Individuals were sampled opportunistically across much of the range of *Bufo nebulifer*, in Texas, USA and Tamaulipas, Mexico, from 1998 to 2007 (Appendix A). In three areas of Texas (Kickapoo Cavern State Park [Edwards County], Griffith League Ranch, and Bastrop State Park [Bastrop County]) multi-year trapping studies were conducted during which tissue was collected (Forstner and Swannack 2004; Jones 2006).

Tissue sampling was non-consumptive where possible. Toe clip or blood tissue samples were collected from live adult toads (muscle or liver was taken from vouchered animals), and some tadpole tails were sampled. Blood samples were stored at –80 °C in a blood storage buffer modified from Longmire et al. (1988): 100 mM TRIS, 100 mM

EDTA disodium dihydrate, 1 % w/v sodium dodecyl sulfate, pH = 8.0. Toe clips, muscle, liver, and tadpoles were stored in 96 % ethanol at –80 °C. Tissues and vouchered specimens were deposited in the Michael R. J. Forstner Frozen Tissue Catalog at Texas State University—San Marcos.

Bufo nebulifer were sampled under Texas Parks and Wildlife Scientific Permit Numbers SPR-0102-191 and SPR-0290-022, Texas Parks and Wildlife Natural Resources Program 25-00, CITES Permit Number 05US704066/9, Costa Rica MINAE Resolucion Numbers 237-98-OFAU and 019-2000-OFAU (Collecting Licenses 0023073 and 0205-00), and Institutional Animal Care and Use Committee approvals 04-3D2AAE71, 0715_0428_07, and 5Qrs45_02.

DNA extraction.—DNA was isolated as described in Chapter 2.

Sequences.—A ~250 base pair (bp) fragment of the control region (D-loop) of the mitochondrial genome (mtDNA) was sequenced. Amplification was performed using the primers BVDL (5'-TCATTTCAATCATTCAAGTGATTT-3') and BUFOR1 (5'-CTGAGGCCGCTTTAAGGTACGATAG-3') in reactions with 4 mmol $MgCl_2$, 0.1 mM dNTPs, 0.01 μM each primer, 2.5 units *Taq* polymerase, and pH = 8.5. PCR was performed with an initial denaturing period of 95 °C for 5 min then 35 cycles, each consisting of denaturing at 95 °C for 30 sec, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, and a final extension period of 72 °C for 5 min. Positive and negative controls were used. PCR products were purified with an AMPure® PCR Purification System (Agencourt Bioscience Corporation), and then cycle sequenced with the above primers, using a CEQ™ DTCS Quick Start Kit (Beckman Coulter) following

manufacturer's instructions. Thermal cycling was 30 cycles of 96 °C for 20 sec, 50 °C for 20 sec, and 60 °C for 4 min. Products were cleaned by ethanol precipitation (following Beckman Coulter manufacturer's instructions) and analyzed on a CEQ™ 8800 Genetic Analysis System (Beckman Coulter). Resultant sequences were edited and aligned in SEQUENCHER™ Version 4.5 (Gene Codes Corp.).

Microsatellites.—Amplifications of microsatellite loci were performed using WellRED fluorescently labeled forward primers (see Table 1) in 10 μl reactions with 4 mmol $MgCl_2$, 0.1 mM dNTPs, 0.01 μM each primer, 2.5 units *Taq* polymerase, and pH = 8.5. PCR was performed with an initial denaturing period of 95 °C for 5 min then 35 cycles, each consisting of denaturing at 95 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, and a final extension period of 72 °C for 5 min. Amplification products were electrophoresed singly on a CEQ™ 8800 Genetic Analysis System (Beckman Coulter) following manufacturer's instructions. Allele sizes were determined with CEQ™ 8800 FRAGMENT ANALYSIS software (Beckman Coulter) by eye. At least two PCR attempts were made, for each individual per locus, before scoring the locus as not amplifiable. See also Chapter 5.

Phylogenetic analyses.—To assess the phylogenetic placement of *B*. *nebulifer*, maximum parsimony (MP), maximum likelihood (ML; Felsenstein 1981), and Bayesian analyses using mtDNA data were performed. *Bufo melanochlorus* (a female from Costa Rica, TCWC84123) was used as an outgroup, and one *Bufo valliceps* (a juvenile from Costa Rica, UCR15722) was also included. Maximum parsimony topologies were generated using equal character weighting, Fitch parsimony, ACCTRAN optimization,

heuristic search, random stepwise addition sequence (10,000 replicates), tree bisection-reconnection (TBR) branch swapping, and MulTrees in PAUP* version 4.0b10 (Swofford 2002). Multiple equally parsimonious trees were summarized using strict consensus. Model parameters for maximum likelihood, which were estimated by hLRT and AIC using MODELTEST version 3.7 (Posada and Crandall 1998), were used as input in a ML heuristic search in PAUP* version 4.0b10 (Swofford 2002). Bootstrap values (Felsenstein 1985) were estimated from 100 replicates in a heuristic search with random stepwise addition sequence (ten replicates) and TBR branch swapping in PAUP* version 4.0b10 (Swofford 2002) for MP and ML analyses. Parameters of a best-fit nucleotide model of evolution for Bayesian analysis were determined by hLRT and AIC in MRMODELTEST version 2.0 (Nylander 2004), and MRBAYES version 3.1.2 (Ronquist and Huelsenbeck 2003) was implemented for ten million generations, saving every thousandth tree, and with a burn-in of 2,500 trees.

To assess intraspecific relationships, a statistical parsimony network (Templeton et al. 1992) of mtDNA haplotypes in *B. nebulifer* was constructed using TSC version 1.21 (Clement et al. 2000).

Genetic clustering analyses.—GENELAND version 3.1.4 (Guillot et al. 2005b; Guillot et al. 2005a; Guillot 2008; Guillot et al. 2008) was used to infer the number of clusters (*K*), or populations, in the dataset and to assign individuals to a cluster as described in Chapter 2. A comparison of genetic clustering analyses is presented in Table 2. The analysis of the dataset that included all individuals ($n = 596$) was analysis A. A similar analysis was also run (analysis B) where the spatial model was not used. Some

loci have many missing data, even after multiple attempts at PCR; to assess whether the results were biased by missing data, a subset of individuals (those with no missing data) was analyzed as above. To determine if GENELAND was detecting only the uppermost hierarchical level of genetic structure, second- and third- order analyses were performed (analyses C, E, G, and I; see Table 2); similar analyses were also run (analyses D, F, H, and J) where the spatial model was not used.

STRUCTURE version 2.1 (Pritchard et al. 2000) was used to infer the number of clusters (*K*), or populations, in the dataset and to assign individuals to a cluster as described in Chapter 2. Values of *K* 1 to 5 were used in all analyses. Falush et al. (2003) suggest using the admixture model and correlated allele frequencies model in situations where there is weak or subtle population structure, which is the most likely scenario in *B. nebulifer* because they are not known to move large distances (Mendelson 2005). The analysis of the dataset that included all individuals ($n = 596$) was analysis K (Table 2). Some loci have missing data, even after multiple attempts at PCR; to assess whether the results were biased by missing data, a subset of individuals (those with no missing data) was analyzed as above. To determine if STRUCTURE was detecting only the uppermost hierarchical level of genetic structure, second- and third-order analyses (analyses L–O; see Table 2) were performed (Evanno et al. 2005).

Genetic diversity analyses.—Allele frequencies, number of private alleles (A_p), and allelic richness (*R*) were estimated using FSTAT version 2.9.3 (Goudet 2001). For allelic richness, FSTAT uses a rarefaction method to adjust for differences in sample sizes (El Mousadik and Petit 1996). Exact tests for Hardy-Weinberg equilibrium (HWE)

were performed with 1,000,000 Markov chain steps and 100,000 dememorization steps in ARLEQUIN version 3.11 (Excoffier et al. 2005). Tests for linkage disequilibrium (LDE) among loci, within or among samples, were performed in FSTAT version 2.9.3 with 1,080 permutations. Significance, of HWE and of LDE, was determined after sequential Bonferroni correction with $\alpha = 0.05$ (Rice 1989).

Differences in allele frequencies among nine groups of sites (identified via multiple methods: genetic clustering analyses and other genetic diversity analyses) were assessed by computing pairwise F_{ST} values in ARLEQUIN version 3.11 (Excoffier et al. 2005) with 10,000 permutations and a significance value of 0.05: groups Cameron (n = 21), HAR01s (n = 7), HAR02s (n = 10), Hill (n = 15), Liberty (n = 19), MEX04s (n = 1), other Mexico sites (n = 16), Terrell & Val Verde (n = 4), and cluster X (n = 503).

Using the microsatellite dataset, isolation-by-distance was tested among individuals with a Mantel test (Mantel 1967) in ALLELES IN SPACE version 1.0 (AIS, Miller 2005). Three analyses were performed, with 10,000 permutations each:

1) all individuals (n = 596)
2) only individuals from cluster O in GENELAND analysis A (n = 65)
3) only individuals from cluster X in GENELAND analysis A (n = 503)

Migration rates.—Migration rates were estimated using a Bayesian, assignment test-based method, as implemented in BAYESASS version 1.3 (Wilson and Rannala 2003) in three analyses: nine groups of sites landscape-wide, 19 groups of sites landscape-wide, and 19 sites at the Griffith League Ranch in Bastrop County. BAYESASS requires <20 populations; consequently, groups of sites were constructed based on geographic locality

and results from GENELAND analyses (Table 3). Initial analyses were performed first to determine the appropriate run length (where convergence of log-likelihood values had been reached) and then to determine the appropriate delta values for allele frequencies (P), migration rates (m), and inbreeding coefficients (F) (40–60 % change in parameter values) (Wilson and Rannala 2003). Once these values were established, ten runs were performed, each with a different starting seed (60, 12, 55, 88, 33, 59, 29, 37, 71, 99), but all with the following input values: iterations = 3,000,000, burn-in = 1,000,000, sampling frequency = 2,000, P = 0.5, m = 0.15, and F = 0.5 (for the analysis of nineteen groups: P = 0.5, m = 0.2, and F = 0.95; and Griffith League Ranch: P = 0.9, m = 0.3, F = 0.99). Distributions of log-likelihood values were compared across runs; the run with the narrowest distribution was used to assess migration rates. Migration rates from all ten runs were compared to see if they converged on a similar solution.

For all analyses, individuals were categorized as 'resident' if assigned ≥800 times to its own group at time 0, 'immigrant' if assigned ≥800 times to another group at time 1, 'progeny of immigrant' if assigned ≥800 times to another group at time 2, or 'non-resident' if not assigned to any one group or time ≥800 times. Additionally, if all individuals in a group were assigned to another group at time 0, then they were categorized as resident and those groups were determined to be indistinct (i.e., they should not have been analyzed as separate groups).

The proportion of males that were resident was compared to the proportion of females that were resident (proportion of juveniles was also compared to that of adults). The test statistic was calculated as: $Z = \frac{\hat{p}_1 - \hat{p}_2}{SE_{H_o}(\hat{p}_1 - \hat{p}_2)}$, where $\hat{p}_1$ = proportion of one

group that were resident, $\hat{p}_2$ =proportion of other group that were resident, $SE_{H_o}(\hat{p}_1 - \hat{p}_2) = \sqrt{\hat{p}(1-\hat{p})(1/n_1 + 1/n_2)}$, n_1 = total number of one group, and n_2 = total number of other group. The confidence interval (CI) for p_1-p_2 was calculated as: $\hat{p}_1 - \hat{p}_2 \mp z_{1-\alpha/2} \bullet \sqrt{\hat{p}_1(1-\hat{p}_1)/n_1 + \hat{p}_2(1-\hat{p}_2)/n_2}$, where $\hat{p} = \frac{x_1 + x_2}{n_1 + n_2}$, x_1 = number of one group that were resident, and x_2 = number of other group that were resident.

AMOVA analyses.—The population genetic structure was examined using a nested hierarchical analysis of molecular variance (AMOVA) for three strategies using microsatellite data:

1) among groups identified by GENELAND analysis A (clusters O and X; individuals with partial memberships in multiple clusters were excluded from the dataset)
2) among nine groups detected via multiple methods (Cameron, HAR01s, HAR02s, Hill, Liberty, MEX04s, other Mexico sites, Terrell & Val Verde, and cluster X)
3) among nineteen groups detected via multiple methods (Cameron; HAR01s; HAR02s; Hill; Liberty; MEX04s; other Mexico sites; Terrell & Val Verde; Aransas; Austin & Colorado; Bandera; Bastrop, Lee, & Travis; Edwards; Ft. Bend; Guadalupe & Hays; Kenedy; Lavaca; Leon; and east cluster X)

AMOVAs were performed in ARLEQUIN version 3.11 (Excoffier et al. 2005) and significance was tested using 10,000 permutations.

RESULTS

Sampling.—Five hundred ninety-six *Bufo nebulifer* in 25 Texas counties and in Tamaulipas, Mexico from 1998–2007 were sampled for my study (Fig. 1b, Tables 4–5, Appendix A). Males were encountered more frequently (356, 59.7 %) than females (118, 19.8 %). Twenty-eight juveniles and 42 tadpoles were sampled. The remaining 52 individuals did not have sex recorded. Almost half (245, 41.1 %) were sampled in Bastrop County. Three hundred forty-nine samples were toe clips, 182 blood, 42 tadpole tail, 18 muscle, and five muscle. Twenty-one vouchers were deposited at the Michael R.J. Forstner Frozen Tissue Catalog at Texas State University—San Marcos (MJF10717–MJF10722, MJF10997–MJF10999, MJF11002, MJF11003, MJF11012, MJF11021, MJF11059, MJF11060, MJF11086, MJF11087, MJF11089, MJF11091, MJF11092).

Phylogenetic analyses.—The 267 bp D-loop alignment of 148 individuals (146 *B. nebulifer*) resulted in 12 unique haplotypes (GenBank Accession Nos. HM021119–HM021130). One hundred eighty-eight characters were constant and 45 were parsimony-informative. The model of evolution that best fit the data was HKY+G, as determined by MODELTEST and by MRMODELTEST. The maximum likelihood phylogram is shown in Fig. 2; *B. melanochlorus* was used as the outgroup. MP, ML, and Bayesian analyses resulted in similar topologies. Plotting uncorrected pairwise distance (after excluding uninformative characters) against absolute number of differences did not reveal any saturation (Fig. 3). *Bufo nebulifer* was monophyletic, but relationships among *B. nebulifer* haplotypes were unresolved.

The statistical parsimony network of 10 unique mtDNA haplotypes in 146 *B. nebulifer* is presented in Fig. 4; six mutations differentiate the haplotypes. Six private haplotypes were detected: MF20855 from Brazos County, MF20960 from Aransas County, MF22162 from a site in Tamaulipas, nebE from Cameron County, nebF from Leon County, and nebG from Cameron County (Table 6). The most common haplotype, nebA (n = 101, 69.2 %), was found in all but two counties, Bandera and Washington, and in Tamaulipas.

Genetic clustering analyses.—Results from all GENELAND analyses are summarized in Table 7. For the dataset including all *B. nebulifer* (n = 596) analyzed using the spatial model in GENELAND (analysis A), the modal value for K was 2. Sixty-five individuals were unambiguously assigned to one cluster, cluster O; 503 were unambiguously assigned to another cluster, cluster X; and 28 were assigned partial membership to clusters O and X. For the dataset including all *B. nebulifer* (n = 596) analyzed without the spatial model in GENELAND (analysis B), the modal value for K was 1. For the dataset including individuals for which there were no missing data (n = 355) analyzed using the spatial model, the modal value for K was 2; assignments were consistent with those found in analysis A. Second- and third-order analyses using the spatial model (analyses C, E, G, I), wherein only individuals with an assignment of cluster X were included (n = 503), only individuals with an assignment of cluster O were included (n = 65), only individuals with an assignment of cluster T were included (n = 48), or only individuals with an assignment of cluster M were included (n = 17) resulted in modal K values of 1, 2, 2, and 1 respectively (Table 7); that is, GENELAND detected

only one cluster for ‘cluster M’ and for ‘cluster X’, while for ‘cluster O’ and ‘cluster T’, GENELAND detected two clusters each. Second- and third-order analyses without the spatial model (analyses D, F, H, J) resulted in modal *K* values of 1, 3, 2, and 2 respectively, but did not reveal any meaningful second- or third-order genetic structure (Table 7).

Results from all STRUCTURE analyses are summarized in Table 8. For the dataset including all *B*. *nebulifer* (*n* = 596; analysis K), the most likely number of clusters was 3; some ad hoc measures of Evanno et al. (2005) support this. The highest average log-likelihood for $K = 3$ was –6591 and ΔK was 4.8. Seventy-six individuals were unambiguously assigned to one cluster, cluster X; 184 were assigned partial membership to clusters M and T; seven were assigned partial membership to clusters T and X; and 329 were assigned partial membership to clusters M, T, and X. All three clusters occurred in all counties and Tamaulipas except Hays County where the individual was assigned partial membership to clusters M and T. For the dataset including individuals for which there were no missing data (*n* = 355), the most likely number of clusters was 2; some ad hoc measures of Evanno et al. (2005) support this. The highest average log-likelihood for $K = 2$ was –4383.9 and ΔK was 3.4. One hundred eleven individuals were unambiguously assigned to one cluster; 162 were unambiguously assigned to another cluster; and 82 were assigned partial membership to both clusters. Both clusters occurred in all counties and Tamaulipas except Terrell and Washington counties. Second- and third-order analyses (analyses L, M, N, O), wherein only individuals with an assignment of cluster X were included (*n* = 503), only individuals with an assignment of cluster O were included

(n = 65), only individuals with an assignment of cluster T were included (n = 48), or only individuals with an assignment of cluster M were included (n = 17) resulted in modal K values of 3, 2, 3, and 3 respectively (Table 8); that is, STRUCTURE detected two clusters in 'cluster O' and three clusters each in 'cluster M', 'cluster T', and 'cluster X'. Some ad hoc measures of Evanno et al. (2005) support this. Partial assignments to multiple clusters dominated all four analyses ($n_{\text{cluster M}}$ = 17 [100 %], $n_{\text{cluster O}}$ = 25 [38.5 %], $n_{\text{cluster T}}$ = 48 [100 %], $n_{\text{cluster X}}$ = 329 [65.4 %]).

Genetic diversity analyses.—Characteristics of genetic diversity are presented in Table 9. For four microsatellites, the total number of alleles was 68. Across the nine groups identified via GENELAND, the number of alleles ranged from 6 to 64 and private alleles ranged from 0 to 28. Among loci, the number of alleles ranged from 10 to 29, private alleles ranged from 3 to 18, and allelic richness ranged from 1.525 to 1.794. After sequential Bonferroni correction, only two loci (BBR86 and BC52.10) at two different groups (HAR01s and HAR02s) significantly deviated from HWE. No loci were determined to be in LDE (P = 0.000926; 1,080 permutations).

Pairwise F_{ST} values were calculated for multiple groups of sites. See Tables 10 and 11 for results among the nine groups identified via multiple methods (F_{ST} = 0.023–0.309) and for results among the 19 groups identified via multiple methods (F_{ST} = 0.010–0.406). Pairwise F_{ST} values associated with the Terrell & Val Verde group were generally the highest (0.223–0.309, Table 10; 0.157–0.351, Table 11), while the lowest values were among the groups within 'cluster X' (0.010–0.185, Table 11).

Mantel tests indicated significant positive, but small to moderate, correlations

between genetic distances and geographic distances (i.e., isolation-by-distance) for all analyses (r = 0.0524 to 0.3398; Table 12).

Migration rates.—In the analysis of nine groups (Cameron, HAR01s, HAR02s, Hill, Liberty, MEX04s, other Mexico sites, Terrell & Val Verde, and cluster X; see Table 3), all ten BAYESASS runs converged on similar solutions for migration rates (data not shown). Migration rates from the best run are presented in Table 13; proportion of residents per group ranged 69.0 %–99.8 %. Standard deviations were mostly <0.05; seven (out of 81) were between 0.054 and 0.080. Among the nine groups, migration rates were generally low; immigrants account for >10 % of the population in only two groups: into Cameron from cluster X and into HAR01s from cluster X. Two hundred fifty-seven out of 356 (72.19 %) males were residents, 82 out of 118 (69.49 %) females were residents, and 20 out of 29 (68.97.%) juveniles were residents. While males were more likely than females to be 'resident' (72.19 % vs. 69.49 %), these proportions were not significantly different according to the proportion test (H_0: proportion of males that were residents = proportion of females that were residents; Z = 0.6859 < 1.96 so fail to reject H_0; 95 % CI = -0.001, 0.066). Similarly, the proportion test comparing adults with juveniles (71.37 % vs. 68.97 %) showed that the proportion of adults that were 'resident' was not significantly different from the proportion of juveniles that were 'resident' (H_0: proportion of adults that were residents = proportion of juveniles that were residents; Z = 0.2775 < 1.96 so fail to reject H_0; 95 % CI = -0.001, 0.049).

In the analysis of 19 groups (see Table 3), most BAYESASS runs converged on similar solutions for migration rates; in only three cases did the value differ in other runs

(data not shown). Migration rates from the best run are presented in Table 14; proportion of residents per group ranged 68.7 %–98.9 %. Standard deviations were mostly <0.05; 20 (out of 361) were between 0.051 and 0.080. Migration rates were generally low; immigrants account for >10 % of the population in six groups: other Mexico sites from Cameron; Bastrop, Lee, & Travis from east cluster X; Edwards from east cluster X; Guadalupe & Hays from Ft. Bend; Lavaca from Bastrop, Lee, & Travis; and Leon from east cluster X. One hundred seventeen out of 356 (32.87 %) males were residents, 38 out of 118 (32.20 %) females were residents, and six out of 29 (20.69 %) juveniles were residents. The proportion of males that were 'resident' was not significantly different from the proportion of females that were 'resident' in these 19 groups (H_0: proportion of males that were residents = proportion of females that were residents; $Z = 0.1328 < 1.96$ so fail to reject H_0), but the 95 % CI indicated that the two groups were different ($1.76e^{-5}$, 0.013). The proportion test comparing adults with juveniles (32.70 % vs. 20.69 %) showed that the proportion of adults that were 'resident' was not significantly different from the proportion of juveniles that were 'resident' (H_0: proportion of adults that were residents = proportion of juveniles that were residents; $Z = 1.345 < 1.96$ so fail to reject H_0), but the 95 % CI indicated that the two groups were different (0.015, 0.225).

In the analysis of sites at the Griffith League Ranch in Bastrop County (see Table 3), all BAYESASS runs converged on similar solutions for migration rates (data not shown). Migration rates from the best run are presented in Table 15; proportion of residents per group ranged 72.6 %–97.6 %. Standard deviations were mostly <0.05; 25 (out of 361) were between 0.051 and 0.115. Migration rates were low (<10 %). Sixty out

of 102 (58.82 %) males were residents, eight out of 19 (42.11 %) females were residents, and five out of six (83.33 %) juveniles were residents. The proportion of males that were 'resident' was not significantly different from the proportion of females that were 'resident' in these groups (H_0: proportion of males that were residents = proportion of females that were residents; $Z = 1.349 < 1.96$ so fail to reject H_0), but the 95 % CI indicated that the two groups were different (0.001, 0.333). The proportion test comparing adults with juveniles (56.19 % vs. 83.33 %) showed that the proportion of adults that were 'resident' was not significantly different from the proportion of juveniles that were 'resident' (H_0: proportion of adults that were residents = proportion of juveniles that were residents; $Z = 1.312 < 1.96$ so fail to reject H_0), but the 95 % CI indicated that the two groups were different (0.063, 0.479).

AMOVA analyses.—AMOVA results showed that most of the variance was within sites (84.32 %–88.22 %; Table 16). Among the hierarchical models, the % total variance ranged 5.57–8.15. The hierarchical model with the highest % total variance is that which used nine groups (Cameron, HAR01s, HAR02s, Hill, Liberty, MEX04s, other Mexico sites, Terrell & Val Verde, and cluster X).

DISCUSSION

Phylogeny and haplotype network.—Recent work (Mendelson 1998; Mulcahy and Mendelson 2000; Frost et al. 2006b; Mulcahy et al. 2006) has shown that *Bufo valliceps* sensu lato comprises two species, *Bufo nebulifer* Girard, 1843 (= *Incilius*

nebulifer (Girard, 1843); Mulcahy and Mendelson 2000; Frost et al. 2006a; Frost et al. 2009) and *Bufo valliceps* Wiegmann, 1833 (= *Incilius valliceps* (Wiegmann, 1833); Mulcahy and Mendelson 2000; Frost et al. 2006a; Frost et al. 2009). Results presented here support the division of *B*. *valliceps* sensu lato into two species: individuals of *B*. *nebulifer* formed a monophyletic clade with high support (Fig. 2). Ten haplotypes were recovered in *B*. *nebulifer* (Figs. 2 and 4, Table 6). Uncorrected pairwise distances ranged from 0.004 to 0.019 with an average of 0.009 in *B*. *nebulifer*.

Mulcahy and Mendelson (2000) estimate the time of divergence of *B*. *nebulifer* and *B*. *valliceps* at 7.6 to 4.2 million years ago (mya). Using a rate of 1.644 % divergence per lineage (3.288 % pairwise) per million years for the D-loop (Stöck et al. 2006), the estimated date of divergence from these data is 7.071 to 6.867 mya (Table 17). While the use of molecular clocks is contested at best (Maxson 1984; Hillis 1987; Moritz et al. 1987; Holder and Lewis 2003; Stöck et al. 2006; Goebel et al. 2009), these estimated divergence dates are consistent with both Mulcahy and Mendelson's (2000) estimate and with the Miocene–Pliocene formation of the Trans-Mexican Neovolcanic Belt (TMNB) (Rosen 1978; de Cserna 1989), which has been hypothesized to be the vicariant event responsible for the separation of the *B*. *nebulifer* and *B*. *valliceps* lineages (Mulcahy and Mendelson 2000).

Of the 10 *B*. *nebulifer* haplotypes, only one was particularly frequent and it accounted for 69.2 % of those sequenced, nebA (n = 101, Figs. 2 and 4, Table 6). The most geographically widespread haplotypes were nebA (Hill Co. to Tamaulipas and Terrell Co. to Liberty Co.), nebB (Brazos Co. to Tamaulipas), and nebC (Edwards Co. to

Burleson Co.; see Table 6). When the samples were grouped into three geographic regions — where the 'northern' region is cluster T and HAR01s, central is cluster X, and southern is cluster M, MEX04, and Cameron Co. — the central and southern regions had more haplotypes (123 individuals yielded seven haplotypes and 13 yielded five, respectively) than did the 'northern' region (10 individuals yielded two haplotypes; Fig. 5). Other workers in this species have found a similar pattern and attributed it to *B. nebulifer* originating in northern Veracruz (just south of the state of Tamaulipas) via the Miocene–Pliocene formation of TMNB and colonizing northward (Mulcahy and Mendelson 2000; Mulcahy et al. 2006; Vogel 2007). Congruent with other bufonid studies (Masta et al. 2002; Pauly et al. 2004; Martínez-Solano and González 2008; Zhang et al. 2008), there is evidence for reticulate relationships among populations of *B. nebulifer* (Fig. 4).

Number of populations in B. nebulifer.—This study is the first to assess population genetic structure at either the landscape or the fine (i.e., habitat patch) scale in *B. nebulifer*. Four polymorphic loci in 596 samples from 169 sites in 25 Texas counties and Tamaulipas (Tables 4, 5, 9) were analyzed using the genetic clustering software GENELAND and STRUCTURE to determine the number of populations. Only results from GENELAND analyses revealed meaningful patterns of genetic structure. Perhaps including more loci would improve the results from STRUCTURE analyses. Missing data, deviation from HWE, and LDE were not determined to influence the results sufficiently to warrant excluding any loci from analyses. Results from analyses A–J suggest that there are nine clusters at different levels of divergence (see also Fig. 1b): Cameron, HAR01s, HAR02s,

Hill, Liberty, MEX04s, other Mexico sites, Terrell & Val Verde, and cluster X. Although Liberty Co. grouped with Terrell and Val Verde counties and HAR02s and Hill Co. clustered together in analyses G, Liberty Co. is likely independent from the far western cluster, and HAR02s and Hill Co. are likely independent from each other, given the geographical distances (>600 km and >300 km respectively).

Although both low and high pairwise F_{ST}values occur at both small and large geographic distances, they do support the delineation of these nine groups (Table 11). Overall, differentiation was greater among geographically disparate groups. This result, increasing F_{ST} with increasing geographic distance but some high F_{ST} values at smaller distances, is consistent with other studies on pond-breeding bufonids (e.g., Rowe et al. 2000; Brede and Beebee 2004; Martínez-Solano and González 2008). Mantel tests indicate little isolation-by-distance within cluster X (0.0524, Table 12) and stronger isolation-by-distance within cluster O (0.3398, Table 12); given the high F_{ST} at the landscape scale, this result is not surprising. Migration rates also corroborate clustering results with little to no migration among the nine groups. In only three cases did immigration exceed 10 % (from cluster X into Cameron, from cluster X into HAR01s, and from Cameron into other Mexico sites; Table 13) and in these cases some migration was expected because both Cameron and HAR01s were assigned partial memberships to clusters X and O in GENELAND analysis A (Table 7). Contrary to genetic clustering analyses, pairwise F_{ST} values, and migration rates, AMOVAs strongly indicated that, regardless of how populations or groups were delineated, little variation was explained at this level (5.57 %–8.15 %), and most of the variation was within sites (84.32 %–88.22 %,

Table 16).

Migration/dispersal.—Generally, very low levels of migration occurred over the entire range of extant *B*. *nebulifer* (Tables 13–15). At distances greater than 10 km, little migration is expected, but almost no migration was seen even at distances ≤4 km (Table 15). For example, BAN02p and BAN04p are <1 km apart, yet 97.6 % and 88.7 % of the individuals sampled, respectively, were residents at each pond (Table 15). Individual toads have been shown to move distances of 700 m in a week and 1.1 km in a few days (a year later, the latter toad was found 1.4 km away at yet another pond; Thornton 1960). However, regular movements of most *B*. *nebulifer* seem to be confined to 50–100 m (Thornton 1960; Awbrey 1963). Larger long-range movement has been documented in other bufonids: 4 km for *B*. *americanus* (Maynard 1934), 6 km in 4 yr for *B*. *boreas* (Muths et al. 2003), 1.6 km in several weeks for *B*. *bufo* (Sinsch 1988), 2.6 km in a breeding season for *B*. *calamita* (Sinsch 1992), 2 km in 2 yr for *B*. *fowleri* (Breden 1987), and 500–2,000 m in *B*. *houstonensis* (Price 1992; Forstner et al. 2003; Price 2003). However, in *B*. *quercicus*, Greenberg and Tanner (2005) found that very few toads move between breeding ponds and when they did it was only 132 m. Given the genetic results here, *B*. *nebulifer* appears to be one of the more sedentary bufonid toads.

Juveniles are the dispersal life-stage in bufonids such as *B*. *bufo*, *B*. *calamita*, and *B*. *fowleri* (Breden 1987; Scribner et al. 1997; Sinsch 1997) and there is some indication of this in *B*. *nebulifer* (Blair 1953; Grubb 1973a), but I found no evidence for it. The proportion of residents in adults was not significantly different from juveniles (Z = 0.2775, 1.345, and 1.312); that is, juveniles either moved at the same frequency or moved

the same distances as adults. Similarly, the proportion of male residents was not different from females (Z = 0.6859, 0.1328, and 1.349). Typical movement distances and long-distance dispersals attainable by *B. nebulifer* correspond to the migration rates found here.

Phylogeography of Bufo nebulifer.—Two major groups within *B. nebulifer*, in Tamaulipas and in Texas, correspond to groups found by Mulcahy and Mendelson (2000) and Mulcahy et al. (2006). The boundary between these two groups remains unknown but may be near the USA-Mexico border (see also Fig. 1) though some gene flow between the two divisions does occur (e.g., partial assignment of individuals from Cameron to clusters O and X). Erratic precipitation and drought in the Lower Rio Grande Valley (Brush 2005) may affect toad intrapopulation demographics and may decrease interpopulation dispersal (Blair 1953). In Texas, four main clusters occur: Terrell & Val Verde in the western reaches of the range, Hill in the north, and HAR01s/HAR02s/Liberty in the east (group names correspond to county of sample origin except the two groups found in Harris County ([HAR01s and HAR02s]). The Terrell & Val Verde cluster is only ~80 km from the nearest site (in Edwards County). Individuals from Edwards County were assigned to a different cluster, cluster X, and high pairwise F_{ST} values and low migration rates support the distinction between Edwards County and Terrell & Val Verde (Table 14). That group Hill is a unique cluster is not surprising, if its geographic isolation is considered (>100 km from the nearest sampling site); however, many sites within cluster X are farther apart but assigned to the same cluster, so geographic distance cannot be the sole reason for the genetic separation of Hill

from other clusters. The eastern set of groups, HAR01s, HAR02s, and Liberty, is located close to cluster X and yet was still discrete from it. Sites HAR01s and HAR02s are located within the urban sprawl of Houston (see also Fig. 1; Radeloff et al. 2005). This alone cannot account for the distinctness of the easternmost *B*. *nebulifer* group, because F_{ST} values indicate that HAR01s and HAR02s are less differentiated from cluster X (0.071, 0.033) than they are from Liberty (0.142, 0.184; Table 10). Migration rates also show higher levels of gene flow between the two sites and cluster X (0.086–0.106) than between the two sites and Liberty (0.065–0.075; Table 13). The city of Houston does appear to negatively affect gene flow, but is not an impenetrable barrier to it. In fact, these latter lines of evidence point to a genetic barrier between cluster X and Liberty that is east of Houston.

One interpretation is that *B*. *nebulifer* has occurred at its present distribution in Texas perhaps as early as 500,000 years ago (Table 17; see also Mulcahy et al. 2006), and its range contracted and subsequently expanded during glacial and interglacial periods. Consequently, individuals in Terrell & Val Verde, Hill, and Liberty may be descendants of disjunct populations from a glacial period when most *B*. *nebulifer* were pushed towards southern Texas. Since the last glaciation, toads re-colonized northwards, and previously disjunct populations came into secondary contact with the southern populations of toads. Phylogeographic breaks in southeastern Texas are hypothesized for *Agkistrodon contortrix* (copperhead; Guiher and Burbrink 2008) and *Elaphe guttata* species complex (cornsnakes; Burbrink 2002). In *A*. *contortrix* the break runs roughly N-S from Fannin County to Matagorda County (Guiher and Burbrink 2008); the

phylogeographic break in *E. guttata* occurs from the northeastern corner of Texas towards Matagorda County (Burbrink 2002). Population expansion out of southern refugia after glacial retreat has been proposed for both snakes; refugia appear to be east of the Trinity River (into Louisiana, possibly to the Mississippi River) and central and/or south Texas for both *A. contortrix* and *E. guttata* (Burbrink 2002; Guiher and Burbrink 2008). A phylogeographic break in west Texas at the Pecos River delineates two main lineages in *Lampropeltis getula* (common kingsnake; Pyron and Burbrink 2009).

For the coastal plain toad, *B. nebulifer*, similar refugia may have occurred in the far western reaches of its range (Terrell & Val Verde), in the north (Hill), in the east (Liberty or possibly farther east like other herpetofauna), and in central Texas and south (cluster X to Tamaulipas). Other authors suggest that the lack of genetic variation in *B. nebulifer*, as compared to *B. valliceps*, results from a recent and rapid colonization northwards (Mulcahy and Mendelson 2000; Mulcahy et al. 2006; Vogel and Johnson 2008). A modified version would postulate that *B. nebulifer* has occurred in much of its present-day range for a long time (at least tens of thousands of years), the 'range-edge' genetic clusters recovered in my study result from glacial refugia of low numbers of toads, and the overall lack of variation in *B. nebulifer* (especially among Texas populations) results from a recent and rapid re-colonization northwards since the last glacial period.

Comparisons with the endangered Bufo houstonensis.—Throughout its range, *B. houstonensis* is sympatric with *B. nebulifer* (Mendelson 2005; Shepard and Brown 2005). Three microsatellite loci (BBR36, BC52.10, and BM224other) amplified in both species

(see also Chapter 2). Similar numbers of alleles per locus were recovered from roughly equal number of individuals in both species for all three loci: 25/421 in *B*. *houstonensis* vs. 19/576 in *B*. *nebulifer* for BBR36, 17/438 vs. 29/370 for BC52.10, and 12/439 vs. 10/595 for BM224other (Table 9; Chapter 2 Table 14). Slightly more mtDNA haplotypes were recovered in *B*. *houstonensis* (14 out of 160 sequenced, Chapter 2 Fig. 4) than were in *B*. *nebulifer* (10 out of 146 sequenced, Table 6), but the D-loop fragment sequenced was ~250 bp smaller in *B*. *nebulifer* so it is likely that more haplotypes could have been found in the latter species with analysis of fragments of equal length. At a comparable geographic scale, migration rates were higher and residency rates were lower in *B*. *houstonensis* (Table 15; see also Chapter 2 Table 20). *Bufo houstonensis* may move farther or more often than do *B*. *nebulifer* because mates are scarcer and must be actively sought in *B*. *houstonensis*, while individual *B*. *nebulifer* are plentiful and are found more easily and proximate. It is also possible that *B*. *houstonensis* are adapted to the canopied habitat found at the Griffith League Ranch (where samples used for these BAYESASS analyses were collected); *B*. *houstonensis* do prefer canopied habitats (Kennedy 1961; Brown 1971; Thomas and Potter 1975; Potter et al. 1984; Sullivan 2005). *Bufo nebulifer* occur in a wide variety of habitats, including woods (Mendelson 2005), but perhaps they are not as adapted to traveling through canopied habitats as well as *B*. *houstonensis*. Movement ability has been shown to vary according to substrate type in other bufonids (Stevens et al. 2004; Stevens et al. 2006).

In conclusion, evidence provided here corroborates previous estimates of divergence dates between *B*. *nebulifer* and *B*. *valliceps* and within *B*. *nebulifer*. My study

is the first to investigate population genetic structure within *B*. *nebulifer*. Multiple clusters, or populations, were recovered from across the range of *B*. *nebulifer*, and their relationships may be explained by a long residence in much of its present-day range, with a history of range contraction (leaving three 'northern' refugia and at least one 'southern' refugium) during glaciation and re-expansion following the retreat of glaciers. Sampling in Veracruz and Louisiana, and additional sampling in some parts of Texas, as well as increasing the number of nuclear loci will improve upon these findings.

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TABLE 1. WellRED fluorescent label, number of alleles (*A*), and size range in bp for four microsatellite loci in *Bufo nebulifer* (*n* = 596).

Locus	Label	*A*	Range	Reference
BBR36	D4	18	153–289	Simandle et al. 2006
BBR86	D4	10	333–366	Simandle et al. 2006
BC52.10	D4	29	155–295	Chan 2007
BM224other	D2	10	60–82	Tikel et al. 2000

TABLE 2. Comparison of genetic clustering analyses.

Analysis	*n*	Spatial model used?	Description
GENELAND			
analysis A	596	yes	all individuals
analysis B	596	no	all individuals
missing data bias	355	yes	individuals with no missing data
analysis C	503	yes	individuals assigned to cluster X in analysis A
analysis D	503	no	individuals assigned to cluster X in analysis A
analysis E	65	yes	individuals assigned to cluster O in analysis A
analysis F	65	no	individuals assigned to cluster O in analysis A
analysis G	48	yes	individuals assigned to cluster T in analysis E
analysis H	48	no	individuals assigned to cluster T in analysis E
analysis I	17	yes	individuals assigned to cluster M in analysis E
analysis J	17	no	individuals assigned to cluster M in analysis E
STRUCTURE			
analysis K	596	n/a	all individuals
missing data bias	355	n/a	individuals with no missing data
analysis L	503	n/a	individuals assigned to cluster X in analysis A
analysis M	65	n/a	individuals assigned to cluster O in analysis A
analysis N	48	n/a	individuals assigned to cluster T in analysis E
analysis O	17	n/a	individuals assigned to cluster M in analysis E

TABLE 3. Groups of sites constructed for three analyses in BAYESASS version 1.3.

BAYESASS Group	County or site	GENELAND analysis A results	GENELAND analysis E results	GENELAND analysis G results
Nine groups landscape-wide				
HAR02s	HAR02s	O	T	T_1
Hill	Hill Co.	O	T	T_1
Liberty	Liberty Co.	O	T	T_2
MEX04s	MEX04s	O	M+T	—
other Mexico sites	other Mexico sites	O	M	—
Terrell & Val Verde	Terrell Co.	O	T	T_2
	Val Verde Co.	O	T	T_2
Cameron	Cameron Co.	O+X	—	—
HAR01s	HAR01s	O+X	—	—
cluster X	Aransas Co.	X	—	—
	Austin Co.	X	—	—
	Bandera Co.	X	—	—
	Bastrop Co.	X	—	—
	Brazos Co.	X	—	—
	Burleson Co.	X	—	—
	Colorado Co.	X	—	—
	Edwards Co.	X	—	—
	Ft. Bend Co.	X	—	—
	Guadalupe Co.	X	—	—
	Hays Co.	X	—	—
	Kenedy Co.	X	—	—
	Lavaca Co.	X	—	—
	Lee Co.	X	—	—
	Leon Co.	X	—	—
	Milam Co.	X	—	—
	Robertson Co.	X	—	—
	Travis Co.	X	—	—
	Washington Co.	X	—	—
Nineteen group landscape-wide				
HAR02s	HAR02s	O	T	T_1
Hill	Hill Co.	O	T	T_1
Liberty	Liberty Co.	O	T	T_2
MEX04s	MEX04s	O	M+T	—
other Mexico sites	other Mexico sites	O	M	—
Terrell & Val Verde	Terrell Co.	O	T	T_2
	Val Verde Co.	O	T	T_2
Cameron	Cameron Co.	O+X	—	—
HAR01s	HAR01s	O+X	—	—
Aransas Co.	Aransas Co.	X	—	—

Austin & Colorado	Austin Co.	X	—	—
	Colorado Co.	X	—	—
Bandera	Bandera Co.	X	—	—
Bastrop, Lee, & Travis	Bastrop Co.	X	—	—
	Lee Co.	X	—	—
	Travis Co.	X	—	—
Edwards	Edwards Co.	X	—	—
Ft. Bend	Ft. Bend Co.	X	—	—
Guadalupe & Hays	Guadalupe Co.	X	—	—
	Hays Co.	X	—	—
Kenedy	Kenedy Co.	X	—	—
Lavaca	Lavaca Co.	X	—	—
Leon	Leon Co.	X	—	—
east cluster X	Brazos Co.	X	—	—
	Burleson Co.	X	—	—
	Milam Co.	X	—	—
	Robertson Co.	X	—	—
	Washington Co.	X	—	—
Nineteen sites at the Griffith League Ranch in Bastrop County, Texas				
BAN02p	BAN02p	X	X	X
BAN12t	BAN12t	X	X	X
BAN14t	BAN14t	X	X	X
BAN23t	BAN23t	X	X	X
BAN04p	BAN04p	X	X	X
BAN15t	BAN15t	X	X	X
BAN24t	BAN24t	X	X	X
BAN07p	BAN07p	X	X	X
BAN11p	BAN11p	X	X	X
BAN38p	BAN38p	X	X	X
BAN17t	BAN17t	X	X	X
BAN32p	BAN32p	X	X	X
BAN37t	BAN37t	X	X	X
BAPp	BAPp	X	X	X
BAN09p	BAN09p	X	X	X
BAN19t	BAN19t	X	X	X
BAN34t	BAN34t	X	X	X
BAN35t	BAN35t	X	X	X
BAN36t	BAN36t	X	X	X

TABLE 4. Numbers of individuals sampled per site by sex and geographic coordinates for each site (latitude and longitude in decimal degrees, WGS84 datum). The terminal letter in a site code represents the type of site: p = pond, s = site, and t = trap.

Site	Latitude	Longitude	Male	Female	Unknown	Total
Aransas County						
ARA01s	28.2403	-96.8344	3	1	1[a]	5
Austin County						
AUS04s	29.88982	-96.3081		1		1
AUS05s	29.89713	-96.3073		1		1
AUS06s	29.87404	-96.3131		1		1
AUS07s	29.87649	-96.3127	4	2		6
AUS08s	29.87681	-96.3125	2			2
AUS09s	29.87718	-96.3121	1	1		2
AUS10s	29.91759	-96.3017	2	1		3
AUS11s	29.8816	-96.3095	1			1
AUS12s	29.89038	-96.3081	1	2		3
AUS13s	29.87515	-96.3126	1			1
Bandera County						
BND01s	29.69726	-99.0506		1		1
BND02s	29.74234	-99.0824	1			1
BND03s	29.80993	-99.5734	1			1
Bastrop County						
BAN02p	30.21626	-97.2417	35	4	2[a], 5[b]	46
BAN04p	30.20932	-97.2429	26			26
BAN07p	30.2056	-97.2342			1[a]	1
BAN09p	30.1978	-97.2133	10			10
BAN11p	30.17795	-97.2338	1	1		2
BAN12t	30.21586	-97.2389	6	1	1[b]	8
BAN14t	30.21658	-97.241	2	2		4
BAN15t	30.21036	-97.2383		1		1
BAN17t	30.21528	-97.2314		1		1
BAN19t	30.2002	-97.2224	1	1		2
BAN23t	30.21586	-97.2393		1		1
BAN24t	30.20953	-97.242		1		1
BAN28p	30.24567	-97.2214	16	1		17
BAN31p	30.36224	-97.256			3[c]	3
BAN32p	30.20997	-97.2482			2[a]	2
BAN33p	30.31756	-97.2678			2[c]	2
BAN34t	30.20014	-97.2217	1		1[a]	2
BAN35t	30.19936	-97.2164	1			1
BAN36t	30.19611	-97.2155		1		1
BAN37t	30.2152	-97.2552		1		1
BAN38p	30.17873	-97.2325	2			2
BAN39s	30.2875	-97.2312		1		1
BAN40s	30.1811	-97.2121			9[b]	9
BAN41p	30.30978	-97.1622			3[c]	3
BAPp	30.19489	-97.2436	17	4		21
BAS01p	30.13288	-97.2657	18			18
BAS08p	30.11438	-97.2767	1			1
BAS09p	30.09016	-97.2385	27			27
BAS14p	30.13941	-97.2512	15			15
BAS15p	30.13721	-97.2434	8	1		9
BAS17p	30.12638	-97.2393	1			1
BAS18p	30.12633	-97.2337	4	1		5
BAS20s	29.96742	-97.3171			1[b]	1

Brazos County						
BRA01s	30.54669	-96.3452			2[a]	2
BRA02s	30.63443	-96.3415			7[a], 1[b]	8
BRA03s	30.39706	-96.2387			1[a]	1
BRA04s	30.78961	-96.2712	4	1		5
BRA05s	30.6505	-96.4671		6		6
Burleson County						
BUR01s	30.41526	-96.6454		2		2
BUR02s	30.57303	-96.6597	1			1
BUR03s	30.57505	-96.6611	1			1
BUR04s	30.59762	-96.6249	1			1
BUR05s	30.59821	-96.5611		1		1
BUR06s	30.60906	-96.6527		1		1
BUR07s	30.60721	-96.6522	1			1
BUR08s	30.59981	-96.6527	1			1
BUR09s	30.5987	-96.6531		1		1
BUR10s	30.6099	-96.6528	1			1
BUR11s	30.58793	-96.5782	1			1
BUR12s	30.39996	-96.6402		1		1
BUR13s	30.4317	-96.7559	1			1
BUR14s	30.39568	-96.6513	1			1
BUR15s	30.54236	-96.7025	1			1
Cameron County						
CAM01p	25.85325	-97.3964	13		6[a], 2[b]	21
Colorado County						
COL02s	29.82978	-96.541		1		1
COL03s	29.85117	-96.5463	1	1		2
COL04s	29.87021	-96.559		1		1
COL05s	29.83003	-96.4803		1		1
COL06s	29.84215	-96.4913		1		1
COL07s	29.8417	-96.4876	1	1		2
COL08s	29.73291	-96.4212	1			1
COL09s	29.73247	-96.4221		1		1
COL10s	29.73353	-96.4199		1		1
COL11s	29.73684	-96.4161		1		1
COL12s	29.72921	-96.4225		1		1
COL13s	29.83018	-96.5219		2		2
COL14s	29.82626	-96.518	1	1		2
COL15s	29.82458	-96.516	1	1		2
COL16s	29.82352	-96.5151	1			1
COL17s	29.82895	-96.5356	1	1		2
COL18s	29.8295	-96.5211	1			1
COL19s	29.90066	-96.58		1		1
COL20s	29.87067	-96.5526	1			1
COL21s	29.74697	-96.4417		1		1
COL22s	29.74102	-96.4469		2		2
COL23s	29.73178	-96.4568	1			1
COL24s	29.71866	-96.4749	1			1
Edwards County						
EDW01s	29.62993	-100.421	6	5	5[a]	16
Ft. Bend County						
FTB01s	29.39735	-95.6251	1	1		2
FTB02s	29.41467	-95.6421			1[a]	1
FTB03s	29.45962	-95.646	3			3
FTB04s	29.45865	-95.646	2			2
FTB05s	29.47257	-95.6458	3		3[a]	6
FTB06s	29.40465	-95.6529	9			9

Guadalupe County						
GUA01p	29.79165	-97.9342			2[a]	2
GUA02t	29.7917	-97.9342		3	2[b]	5
GUA03t	29.79252	-97.9366	1			1
Harris County						
HAR01s	29.68737	-95.503	3	3	1[a]	7
HAR02s	29.71265	-95.3918	6	2	2[a]	10
Hays County						
HAY01s	29.88442	-97.9392		1		1
Hill County						
HIL01s	32.10653	-97.3182	8	4	3[b]	15
Kenedy County						
KEN01s	27.10226	-97.4406		1	2[b]	3
Lavaca County						
LAV01s	29.4469	-97.1794		1		1
LAV02s	29.34291	-96.8386		1		1
LAV03s	29.36116	-96.824	1	2		3
LAV04s	29.44631	-97.1788	1	1		2
LAV05s	29.43923	-97.1692	2	1		3
LAV06s	29.43913	-97.1704	1	1		2
LAV07s	29.43923	-97.1713	1			1
LAV08s	29.45069	-97.1817	3	2		5
LAV09s	29.4469	-97.1779		2		2
LAV10s	29.44631	-97.178	1			1
Lee County						
LEE03p	30.32764	-97.1696			1[c]	1
LEE04p	30.32186	-97.1276	8			8
LEE05p	30.32586	-97.1774			6[c]	6
LEE06p	30.33374	-97.1821			3[c]	3
LEE07p	30.33328	-97.1805			3[c]	3
LEE08s	30.31102	-97.1582	3	1	2[a]	6
LEE09p	30.37143	-97.2453			1[c]	1
LEE10p	30.37764	-97.2477			3[c]	3
LEE11p	30.37191	-97.2111			3[c]	3
LEE12p	30.38786	-97.2009			3[c]	3
LEE13p	30.41608	-97.1837			2[c]	2
LEE14s	30.31797	-97.1217		1		1
LEE15p	30.34278	-97.1843			2[c]	2
LEE16p	30.34596	-97.184			3[c]	3
LEE17p	30.31743	-97.121			4[c]	4
Leon County						
LEOp	31.0775	-96.1933	1			1
LEO02s	31.38305	-95.8062	8	1		9
LEO03s	31.37573	-95.8281	6			6
Liberty County						
LIB01s	30.033	-94.9045	1			1
LIB02s	30.03193	-94.9045	1		2[a]	3
LIB03s	30.02978	-94.9045		1		1
LIB04s	30.02867	-94.9045		1		1
LIB05s	30.02055	-94.9045			1[a]	1
LIB06s	30.01875	-94.9045		2	1[a]	3
LIB07s	30.0257	-94.9044			1[a]	1
LIB08s	30.02695	-94.9045		1		1
LIB09s	30.02867	-94.9045		1		1
LIB10s	30.0306	-94.9045		1		1
LIB11s	30.03165	-94.9045			1[a]	1
LIB12s	30.03248	-94.9044	1	1	1[a]	3
LIB13s	30.03043	-94.9045			1[a]	1

Milam County						
MIL02s	30.72427	-96.8454	1			1
MIL03s	30.71423	-96.8538	2	1		3
MIL04s	30.75997	-96.6834	1			1
MIL05p	30.73318	-96.6778	12	1		13
Robertson County						
ROB01s	30.85636	-96.5369	1			1
ROB02s	30.89853	-96.4357	1			1
ROB03s	31.03957	-96.5956		1		1
Terrell County						
TER01s	30.43611	-101.786		1	2[b]	3
Travis County						
TRA01t	30.3146	-97.7652	1	1		2
Val Verde County						
VAL01s	30.00302	-101.171			1[a]	1
Washington County						
WAS01s	30.18083	-96.5977		1		1
Tamaulipas, Mexico						
MEX01s	23.03964	-98.8323	4			4
MEX02s	23.17525	-98.4359			1[b]	1
MEX03s	23.38668	-99.3933	1			1
MEX04s	24.99848	-98.3027		1		1
MEX05s	23.40495	-99.3695	3			3
MEX06s	23.80923	-98.2647			1[a]	1
MEX07s	23.21861	-98.4575		1		1
MEX08s	23.61883	-99.258		1		1
MEX09s	23.20467	-98.4368	1			1
MEX10s	22.91333	-99.4883			2[a]	2
MEX11s	22.83444	-99.5736		1		1
Totals			356	118	52[a], 28[b], 42[c]	596

[a] Sex not recorded
[b] Juvenile
[c] Tadpole

TABLE 5. Numbers of individuals sampled per county/site by sex and geographic coordinates for each site (latitude and longitude in decimal degrees, WGS84 datum). The terminal letter in a site code represents the type of site: p = pond, s = site, and t = trap.

County & Tamaulipas	Male	Female	Unknown	Total
Aransas Co.	3	1	1[a]	5
Austin Co.	12	9		21
Bandera Co.	2	1		3
Bastrop Co.	192	23	6[a], 16[b], 8[c]	245
Brazos Co.	4	7	10[a], 1[b]	22
Burleson Co.	10	6		16
Cameron Co.	13		6[a], 2[b]	21
Colorado Co.	11	19		30
Edwards Co.	6	5	5[a]	16
Ft. Bend Co.	18	1	4[a]	23
Guadalupe Co.	1	3	2[a], 2[b]	8
Harris Co.				
HAR01s	3	3	1[a]	7
HAR02s	6	2	2[a]	10
Hays Co.		1		1
Hill Co.	8	4	3[b]	15
Kenedy Co.		1	2[b]	3
Lavaca Co.	10	11		21
Lee Co.	11	2	2[a], 34[c]	49
Leon Co.	15	1		16
Liberty Co.	3	8	8[a]	19
Milam Co.	16	2		18
Robertson Co.	2	1		3
Terrell Co.		1	2[b]	3
Travis Co.	1	1		2
Val Verde Co.			1[a]	1
Washington Co.		1		1
Mexico				
MEX04s		1		1
other sites	9	3	3[a], 1[b]	16
Totals	356	118	52[a], 28[b], 42[c]	596

[a] Sex not recorded
[b] Juvenile
[c] Tadpole

TABLE 6. Mitochondrial (mtDNA) haplotypes found per county/site. Number of individuals sequenced (*n*) and percent sequenced of total sampled (%) are provided.

County or site (*n*, %)	mtDNA haplotype									
	nebA	nebB	nebC	nebD	nebE	nebF	nebG	MF20855	MF20960	MF22162
Aransas Co. (4, 80 %)	3								1	
Austin Co. (1, 4.8 %)	1									
Bandera Co. (2, 66.7 %)			1	1						
Bastrop Co. (41, 16.7 %)	32		8	1						
Brazos Co. (4, 18.2 %)	2	1						1		
Burleson Co. (2, 12.5 %)	1		1							
Cameron Co. (8, 38.1 %)	3				3		2			
Colorado Co. (4, 13.3 %)	4									
Edwards Co. (6, 37.5 %)	5		1							
Ft. Bend Co. (2, 8.7 %)	2									
Guadalupe Co. (5, 62.5 %)	5									
Harris Co.										
HAR01s (1, 14.3 %)	1									
HAR02s (1, 10 %)	1									
Hays Co. (1, 100 %)	1									
Hill Co. (2, 13.3 %)	2									
Kenedy Co. (3, 100 %)	3									
Lavaca Co. (2, 9.5 %)	1		1							
Lee Co. (36, 73.5 %)	20	10	2	4						
Leon Co. (3, 18.8 %)	2					1				
Liberty Co. (2, 10.5 %)	1	1								
Milam Co. (2, 11.1 %)	2									
Robertson Co. (2, 66.7 %)	1			1						
Terrell Co. (3, 100 %)	3									
Travis Co. (2, 100 %)	2									
Val Verde Co. (1, 100 %)	1									
Washington Co. (1, 100 %)			1							
Mexico										
MEX04s (0, 0 %)										
other sites (5, 31.3 %)	2	2								1
Totals (146, 24.5 %)	101	14	15	7	3	1	2	1	1	1

TABLE 7. Summary of GENELAND version 3.1.4 results per county/site by analysis. Number of clusters found for each analysis is below analysis name. See also Table 2 for descriptions of analyses.

County or site	No. of sites	*n*	GENELAND analysis								
			A *K* = 2	B *K* = 1	C & D *K* = 1	E *K* = 2	F *K* = 3	G *K* = 2	H *K* = 2	I *K* = 1	J *K* = 2
Aransas Co.	1	5	X	X	X	—	—	—	—	—	—
Austin Co.	10	21	X	X	X	—	—	—	—	—	—
Bandera Co.	3	3	X	X	X	—	—	—	—	—	—
Bastrop Co.	33	245	X	X	X	—	—	—	—	—	—
Brazos Co.	5	22	X	X	X	—	—	—	—	—	—
Burleson Co.	15	16	X	X	X	—	—	—	—	—	—
Cameron Co.	1	21	O+X	X	—	—	—	—	—	—	—
Colorado Co.	23	30	X	X	X	—	—	—	—	—	—
Edwards Co.	1	16	X	X	X	—	—	—	—	—	—
Ft. Bend Co.	6	23	X	X	X	—	—	—	—	—	—
Guadalupe Co.	3	8	X	X	X	—	—	—	—	—	—
Harris Co.	2										
HAR01s		7	O+X	X	—	—	—	—	—	—	—
HAR02s		10	O	X	—	T	T_1	T_1	T_1	—	—
Hays Co.	1	1	X	X	X	—	—	—	—	—	—
Hill Co.	1	15	O	X	—	T	T_1	T_1	T_1	—	—
Kenedy Co.	1	3	X	X	X	—	—	—	—	—	—
Lavaca Co.	10	21	X	X	X	—	—	—	—	—	—
Lee Co.	15	49	X	X	X	—	—	—	—	—	—
Leon Co.	3	16	X	X	X	—	—	—	—	—	—
Liberty Co.	13	19	O	X	—	T	$M+T_1+T_2$	T_2	T_1+T_2	—	—
Milam Co.	4	18	X	X	X	—	—	—	—	—	—
Robertson Co.	3	3	X	X	X	—	—	—	—	—	—
Terrell Co.	1	3	O	X	—	T	T_2	T_2	T_2	—	—
Travis Co.	1	2	X	X	X	—	—	—	—	—	—
Val Verde Co.	1	1	O	X	—	T	T_1+T_2	T_2	T_2	—	—
Washington Co.	1	1	X	X	X	—	—	—	—	—	—
Mexico	11										
MEX04s		1	O	X	—	M+T	T_1	—	—	—	—
other sites		16	O	X	—	M	$M+T_1+T_2$	—	—	M	M_1+M_2

TABLE 8. Summary of STRUCTURE version 2.1 results per county/site by analysis. Number of clusters found for each analysis is below analysis name. See also Table 2 for descriptions of analyses.

County or site	No. of sites	*n*	STRUCTURE analysis				
			K *K* = 3	L *K* = 3	M *K* = 2	N *K* = 3	O *K* = 3
Aransas Co.	1	5	M+T+X	X_2	—	—	—
Austin Co.	10	21	M+T+X	$X_1+X_2+X_3$	—	—	—
Bandera Co.	3	3	M+T+X	$X_1+X_2+X_3$	—	—	—
Bastrop Co.	33	245	M+T+X	$X_1+X_2+X_3$	—	—	—
Brazos Co.	5	22	M+T+X	$X_1+X_2+X_3$	—	—	—
Burleson Co.	15	16	M+T+X	$X_1+X_2+X_3$	—	—	—
Cameron Co.	1	21	M+T+X	—	—	—	—
Colorado Co.	23	30	M+T+X	$X_1+X_2+X_3$	—	—	—
Edwards Co.	1	16	M+T+X	$X_1+X_2+X_3$	—	—	—
Ft. Bend Co.	6	23	M+T+X	$X_1+X_2+X_3$	—	—	—
Guadalupe Co.	3	8	M+T+X	$X_1+X_2+X_3$	—	—	—
Harris Co.	2						
HAR01s		7	M+T+X	—	—	—	—
HAR02s		10	M+T+X	—	T_1+T_2	$T_1+T_2+T_3$	—
Hays Co.	1	1	M+T	X_3	—	—	—
Hill Co.	1	15	M+T+X	—	T_1+T_2	$T_1+T_2+T_3$	—
Kenedy Co.	1	3	M+T+X	$X_1+X_2+X_3$	—	—	—
Lavaca Co.	10	21	M+T+X	$X_1+X_2+X_3$	—	—	—
Lee Co.	15	49	M+T+X	$X_1+X_2+X_3$	—	—	—
Leon Co.	3	16	M+T+X	$X_1+X_2+X_3$	—	—	—
Liberty Co.	13	19	M+T+X	—	T_1+T_2	$T_1+T_2+T_3$	—
Milam Co.	4	18	M+T+X	$X_1+X_2+X_3$	—	—	—
Robertson Co.	3	3	M+T+X	$X_1+X_2+X_3$	—	—	—
Terrell Co.	1	3	M+T+X	—	T_1+T_2	$T_1+T_2+T_3$	—
Travis Co.	1	2	M+T+X	$X_1+X_2+X_3$	—	—	—
Val Verde Co.	1	1	M+T+X	—	T_1	$T_1+T_2+T_3$	—
Washington Co.	1	1	M+T+X	X_2+X_3	—	—	—
Mexico	11						
MEX04s		1	M+T	—	T_1+T_2	—	$M_1+M_2+M_3$
other sites		16	M+T+X	—	T_1+T_2	—	$M_1+M_2+M_3$

TABLE 9. Characteristics of genetic diversity in nine groups identified via GENELAND version 3.1.4. Sample size (n), number of alleles (A), number of private alleles (A_p), allelic richness (R, based on a minimum sample size of 1), and expected (H_E) and observed (H_E) heterozygosities are provided. Observed heterozygosities followed by a * significantly deviated from HWE before sequential Bonferroni correction, and those in bold significantly deviated after correction.

Locus	Cameron	HAR01s	HAR02s	Hill	Liberty	MEX04s	other Mexico sites	Terrell & Val Verde	cluster X	All
BBR36										
n	18	7	9	15	19	1	16	4	487	576
A	7	3	3	2	3	2	10	2	19	19
A_p	0	0	0	0	0	0	0	0	7	7
R	1.740	1.648	1.523	1.129	1.280	2.000	1.752	1.429	1.819	1.794
H_E	0.739	0.648	0.523	0.129	0.280	1.000	0.752	0.429	0.819	
H_O	0.722	0.429	0.444	0.133	0.316	1.000	0.813	0.000	0.676*	
BBR86										
n	21	7	10	15	19	1	16	4	496	589
A	4	3	3	3	5	1	7	3	9	10
A_p	0	0	0	0	0	0	0	0	3	3
R	1.675	1.604	1.416	1.421	1.717	1.000	1.867	1.464	1.473	1.525
H_E	0.675	0.604	0.416	0.421	0.717	NA	0.867	0.464	0.473	
H_O	0.523	**0.143***	0.300	0.267	0.526	NA	0.875	0.500	0.389*	
BC52.10										
n	12	3	5	15	8	1	3	1	322	370
A	4	3	4	6	6	1	2	1	26	29
A_p	0	0	0	0	2	0	0	1	15	18
R	1.670	1.600	1.733	1.793	1.842	1.000	1.533	1.000	1.728	1.737
H_E	0.670	0.600	0.733	0.793	0.842	NA	0.533	NA	0.728	
H_O	0.250*	0.333*	**0.200***	0.333*	0.250*	NA	0.000	NA	0.366*	
BM224other										
n	21	7	10	15	19	1	16	4	502	595
A	6	7	4	3	7	2	5	3	10	10
A_p	0	0	0	0	0	0	0	0	3	3
R	1.616	1.890	1.684	1.680	1.708	2.000	1.611	1.464	1.732	1.737
H_E	0.616	0.890	0.684	0.680	0.708	1.000	0.611	0.464	0.732	
H_O	0.476*	0.714	0.600	0.867	0.684	1.000	0.563	0.500	0.539*	
Total										
n	21	7	10	15	19	1	16	4	503	596
A	21	16	14	14	21	6	24	9	64	68
A_p	0	0	0	0	2	0	0	1	28	31
Mean H_E	0.675	0.686	0.589	0.503	0.637	1.000	0.691	0.452	0.688	
Mean H_O	0.493	0.405	0.386	0.400	0.444	1.000	0.563	0.333	0.493	

TABLE 10. Pairwise F_{ST} values for nine groups identified by GENELAND version 3.1.4 analyses. Significant F_{ST} values are shown in bold.

Group	Cameron (n = 21)	HAR01s (n = 7)	HAR02s (n = 10)	Hill (n = 15)	Liberty (n = 19)	MEX04s (n = 1)	other Mexico sites (n = 16)	Terrell & Val Verde (n = 4)	cluster X (n = 503)
Cameron	—								
HAR01s	**0.092**	—							
HAR02s	**0.065**	0.031	—						
Hill	**0.149**	**0.183**	**0.107**	—					
Liberty	**0.165**	**0.142**	**0.184**	**0.161**	—				
MEX04s	0.112	0.144	0.227	0.348	0.082	—			
other Mexico sites	0.021	**0.091**	**0.121**	**0.257**	**0.190**	0.066	—		
Terrell & Val Verde	**0.228**	**0.252**	**0.309**	**0.262**	0.026	0.126	**0.256**	—	
cluster X	**0.023**	**0.071**	**0.033**	**0.123**	**0.180**	0.086	**0.094**	**0.223**	—

TABLE 11. Pairwise F_{ST} values for nineteen groups identified by multiple methods. Significant F_{ST} values are shown in bold. Grey shading indicates groups of sites in cluster X from GENELAND analysis A.

Group	Cameron (n = 21)	HAR01s (n = 7)	HAR02s (n = 10)	Hill (n = 15)	Liberty (n = 19)	MEX04s (n = 1)	other Mexico sites (n = 16)	Terrell & Val Verde (n = 4)	cluster X: Aransas (n = 5)	cluster X: Austin & Colorado (n = 51)
Cameron	—									
HAR01s	**0.092**	—								
HAR02s	**0.065**	0.031	—							
Hill	**0.149**	**0.183**	**0.107**	—						
Liberty	**0.165**	**0.142**	**0.184**	**0.161**	—					
MEX04s	0.112	0.144	0.227	0.348	0.082	—				
other Mexico sites	0.021	**0.091**	**0.121**	**0.257**	**0.190**	0.066	—			
Terrell & Val Verde	**0.228**	**0.252**	**0.309**	**0.262**	0.026	0.126	**0.256**	—		
Aransas	-0.030	0.030	0.031	**0.162**	**0.156**	0.041	0.003	**0.246**	—	
Austin & Colorado	0.018	**0.057**	**0.039**	**0.148**	**0.181**	0.077	**0.068**	**0.220**	-0.020	—
Bandera	0.012	0.021	0.069	**0.231**	**0.247**	0.114	0.056	**0.325**	0.001	0.008
Bastrop, Lee, & Travis	**0.035**	**0.081**	**0.039**	**0.141**	**0.203**	0.112	**0.108**	**0.245**	-0.003	**0.010**
east cluster X	**0.024**	**0.083**	0.022	**0.080**	**0.188**	0.168	**0.117**	**0.253**	-0.013	**0.029**
Edwards	**0.108**	**0.100**	**0.118**	**0.250**	**0.220**	0.059	**0.140**	**0.225**	0.061	**0.040**
Ft. Bend	0.025	**0.068**	0.009	**0.079**	**0.161**	0.112	**0.107**	**0.230**	-0.020	0.019
Guadalupe & Hays	**0.155**	**0.179**	**0.231**	**0.294**	**0.145**	-0.168	**0.168**	**0.157**	0.091	**0.119**
Kenedy	**0.129**	0.112	**0.218**	**0.406**	**0.289**	0.165	**0.082**	**0.351**	0.086	0.063
Lavaca	**0.035**	**0.083**	**0.083**	**0.171**	**0.158**	-0.031	**0.082**	**0.181**	-0.019	**0.023**
Leon	0.014	**0.157**	**0.123**	**0.171**	**0.188**	0.087	**0.063**	**0.262**	0.010	**0.065**

TABLE 11 continued.

Group	cluster X								
	Bandera (*n* = 3)	Bastrop, Lee, & Travis (*n* = 296)	east cluster X (*n* = 60)	Edwards (*n* = 16)	Ft. Bend (*n* = 23)	Guadalupe & Hays (*n* = 9)	Kenedy (*n* = 3)	Lavaca (*n* = 21)	Leon (*n* = 16)
Cameron									
HAR01s									
HAR02s									
Hill									
Liberty									
MEX04s									
other Mexico sites									
Terrell & Val Verde									
Aransas									
Austin & Colorado									
Bandera	—								
Bastrop, Lee, & Travis	-0.008	—							
east cluster X	0.036	**0.030**	—						
Edwards	0.031	**0.037**	**0.121**	—					
Ft. Bend	0.042	**0.026**	0.003	**0.098**	—				
Guadalupe & Hays	**0.183**	**0.145**	**0.184**	**0.108**	**0.133**	—			
Kenedy	0.137	**0.118**	**0.185**	0.100	**0.157**	0.179	—		
Lavaca	-0.005	**0.018**	**0.053**	0.025	**0.039**	**0.062**	**0.117**	—	
Leon	0.075	**0.084**	**0.056**	**0.168**	**0.059**	**0.160**	**0.202**	**0.059**	—

TABLE 12. Summary of results from Mantel tests, as calculated in AIS version 1.0. For each dataset, regressions were performed on geographic distances. Number of samples (*n*), correlation coefficient (*r*), and significance value (*P*) are provided.

Analysis	*n*	*r*	*P*
All individuals	596	0.0965	<0.0001
Only individuals from cluster O in GENELAND analysis A	65	0.3398	<0.0001
Only individuals from cluster X in GENELAND analysis A	503	0.0524	<0.05

TABLE 13. Migration rates among nine *Bufo nebulifer* groups described in Table 3, obtained using BAYESASS version 1.3.

FROM	INTO								
	Cameron (*n* = 21)	HAR01s (*n* = 7)	HAR02s (*n* = 10)	Hill (*n* = 15)	Liberty (*n* = 19)	MEX04s (*n* = 1)	other Mexico sites (*n* = 16)	Terrell & Val Verde (*n* = 4)	cluster X (*n* = 503)
Cameron	**0.780**[a]	0.025	0.027	0.003	0.007	0.027	**0.204**[a]	0.024	0.000
HAR01s	0.005	**0.705**	0.011	0.002	0.003	0.025	0.008	0.022	0.000
HAR02s	0.006	0.015	**0.696**	0.003	0.003	0.024	0.010	0.022	0.000
Hill	0.014	0.020	0.067	**0.976**	0.008	0.030	0.014	0.036	0.000
Liberty	0.019	0.075	0.065	0.003	**0.961**	0.031	0.037	0.099[a]	0.000
MEX04s	0.005	0.015	0.012	0.003	0.003	**0.780**[a]	0.008	0.022	0.000
other Mexico sites	0.006	0.022	0.023	0.002	0.004	0.026	**0.690**	0.025	0.000
Terrell & Val Verde	0.006	0.016	0.013	0.003	0.003	0.025	0.008	**0.722**	0.000
cluster X	**0.160**[a]	**0.106**[a]	0.086[a]	0.004	0.008	0.033	0.020	0.029	**0.998**

Source groups (FROM) are listed in the left-hand column, receiving groups (INTO) are listed across the top row. Bold values are migration rates >0.1. All values were consistent across ten runs.

[a]Standard deviation was 0.054–0.080. All other standard deviations were <0.05.

TABLE 14. Migration rates among nineteen *Bufo nebulifer* groups described in Table 3, obtained using BAYESASS version 1.3.

FROM	INTO									
									cluster X	
	Cameron (*n* = 21)	HAR01s (*n* = 7)	HAR02s (*n* = 10)	Hill (*n* = 15)	Liberty (*n* = 19)	MEX04s (*n* = 1)	other Mexico sites (*n* = 16)	Terrell & Val Verde (*n* = 4)	Aransas (*n* = 5)	Austin & Colorado (*n* = 51)
Cameron	**0.792**[a]	0.010	0.008	0.002	0.002	0.010	**0.167**[a]	0.011	0.024	0.004
HAR01s	0.003	**0.706**	0.006	0.001	0.002	0.011	0.005	0.011	0.009	0.002
HAR02s	0.003	0.007	**0.698**	0.001	0.002	0.010	0.006	0.010	0.009	0.002
Hill	0.012	0.016	0.049[a]	**0.976**	0.003	0.013	0.006	0.022	0.012	0.003
Liberty	0.006	0.048	0.033	0.001	**0.966**	0.012	0.016	0.056[a]	0.013	0.002
MEX04s	0.003	0.008	0.005	0.001	0.001	**0.783**[a]	0.006	0.009	0.009	0.002
other Mexico sites	0.004	0.018	0.013	0.001	0.002	0.012	**0.696**	0.011	0.013	0.002
Terrell & Val Verde	0.004	0.007	0.006	0.001	0.001	0.010	0.004	**0.738**[a]	0.008	0.002
Aransas	0.003	0.008	0.008	0.001	0.001	0.012	0.005	0.011	**0.714**	0.002
Austin & Colorado	0.009	0.014	0.016	0.001	0.002	0.013	0.018	0.014	0.024	**0.816**[a]
Bandera	0.004	0.007	0.006	0.001	0.002	0.011	0.007	0.010	0.010	0.002
Bastrop, Lee, & Travis	0.006[a]	0.030	0.019	0.001	0.003	0.014	0.010	0.019	0.027	0.038
east cluster X	0.031	0.059[a]	0.059[a]	0.002	0.003	0.015	0.008	0.011	0.033	0.069[a]
Edwards	0.004	0.010	0.006	0.001	0.002	0.012	0.005	0.013	0.009	0.003
Ft. Bend	0.042	0.019	0.042[a]	0.001	0.003	0.013	0.013	0.014	0.043	0.044[a]
Guadalupe & Hays	0.003	0.009	0.006	0.001	0.001	0.010	0.005	0.009	0.009	0.003
Kenedy	0.003	0.007	0.007	0.001	0.002	0.012	0.005	0.008	0.009	0.002
Lavaca	0.005	0.009	0.007	0.001	0.002	0.014	0.007	0.015	0.016	0.002
Leon	0.003	0.008	0.006	0.002	0.001	0.013	0.007	0.010	0.008	0.002

Table 14 continued.

FROM	INTO cluster X								
	Bandera (*n* = 3)	Bastrop, Lee, & Travis (*n* = 296)	east cluster X (*n* = 60)	Edwards (*n* = 16)	Ft. Bend (*n* = 23)	Guadalupe & Hays (*n* = 9)	Kenedy (*n* = 3)	Lavaca (*n* = 21)	Leon (*n* = 16)
Cameron	0.021	0.001	0.001	0.007	0.002	0.008	0.014	0.006	0.031
HAR01s	0.011	0.000	0.000	0.005	0.002	0.007	0.012	0.004	0.005
HAR02s	0.012	0.000	0.001	0.004	0.002	0.008	0.011	0.004	0.005
Hill	0.016	0.001	0.001	0.007	0.005	0.009	0.011	0.013	0.008
Liberty	0.012	0.001	0.001	0.005	0.005	0.011	0.015	0.005	0.009
MEX04s	0.009	0.000	0.000	0.005	0.002	0.007	0.012	0.004	0.004
other Mexico sites	0.011	0.000	0.000	0.006	0.001	0.007	0.015	0.006	0.009
Terrell & Val Verde	0.011	0.000	0.001	0.005	0.001	0.007	0.011	0.003	0.004
Aransas	0.011	0.000	0.001	0.004	0.002	0.007	0.011	0.004	0.005
Austin & Colorado	0.014	0.001	0.001	0.008	0.004	0.026	0.029	0.011	0.028
Bandera	**0.734**[a]	0.000	0.001	0.004	0.001	0.008	0.011	0.005	0.005
Bastrop, Lee, & Travis	0.040	**0.847**	0.001	**0.196**[a]	0.005	0.037	0.026	**0.150**[a]	0.027
east cluster X	0.023	**0.129**	**0.989**	0.011	0.009[b]	0.024	0.020	0.058[a]	**0.140**[ab]
Edwards	0.011	0.001	0.001	**0.702**	0.002	0.007	0.012	0.005	0.004
Ft. Bend	0.016	0.015	0.001	0.008	**0.951**[a]	**0.108**[ab]	0.021	0.022	0.011
Guadalupe & Hays	0.011	0.000	0.000	0.005	0.001	**0.697**	0.011	0.004	0.005
Kenedy	0.011	0.000	0.000	0.004	0.001	0.007	**0.734**[a]	0.004	0.005
Lavaca	0.014	0.001	0.000	0.008	0.001	0.009	0.013	**0.687**	0.005
Leon	0.013	0.000	0.000	0.005	0.002	0.008	0.012	0.004	**0.689**

Source groups (FROM) are listed in the left-hand column, receiving groups (INTO) are listed across the top row. Bold values are migration rates >0.1. The box frames values within cluster X.

[a]Standard deviation was 0.051–0.080. All other standard deviations were <0.05.

[b]Value was <0.1 in all other runs (except from east cluster X into Ft. Bend where half the runs were >0.1). All other values were consistent across all ten runs.

TABLE 15. Migration rates among *Bufo nebulifer* groups at the Griffith League Ranch in Bastrop County described in Table 3, obtained using BAYESASS version 1.3.

	INTO									
			near BAN02p				near BAN04p			
FROM	BAN37t (n = 1)	BAN32p (n = 2)	BAN02p (n = 46)	BAN12t (n = 8)	BAN14t (n = 4)	BAN23t (n = 1)	BAN04p (n = 26)	BAN15t (n = 1)	BAN24t (n = 1)	BAN07p (n = 1)
BAN37t	**0.834**[a]	0.010	0.001	0.005	0.011	0.012	0.002	0.010	0.010	0.012
BAN32p	0.010	**0.762**[a]	0.001	0.005	0.009	0.011	0.002	0.012	0.010	0.011
BAN02p	0.010	0.028	**0.976**	0.023	0.034[a]	0.015	0.038[a]	0.015	0.013	0.016
BAN12t	0.010	0.019	0.003	**0.862**[a]	0.022	0.014	0.023	0.015	0.011	0.014
BAN14t	0.010	0.010	0.001	0.005	**0.726**	0.011	0.002	0.010	0.010	0.012
BAN23t	0.008	0.011	0.001	0.004	0.010	**0.794**[a]	0.002	0.010	0.011	0.011
BAN04p	0.008	0.021	0.006	0.010	0.024	0.015	**0.887**[a]	0.016	0.012	0.015
BAN15t	0.009	0.009	0.001	0.005	0.008	0.011	0.002	**0.786**[a]	0.010	0.012
BAN24t	0.009	0.011	0.001	0.004	0.009	0.010	0.002	0.010	**0.802**[a]	0.012
BAN07p	0.008	0.010	0.001	0.005	0.011	0.009	0.003	0.013	0.010	**0.781**[a]
BAN17t	0.010	0.010	0.001	0.004	0.009	0.010	0.002	0.010	0.013	0.012
BAN09p	0.009	0.012	0.001	0.007	0.012	0.012	0.005	0.012	0.011	0.012
BAN19t	0.010	0.011	0.001	0.004	0.010	0.012	0.003	0.009	0.011	0.013
BAN34t	0.011	0.011	0.001	0.006	0.008	0.011	0.002	0.012	0.011	0.010
BAN35t	0.008	0.012	0.001	0.005	0.009	0.010	0.002	0.010	0.011	0.013
BAN36t	0.009	0.011	0.001	0.004	0.009	0.010	0.003	0.011	0.010	0.011
BAPp	0.010	0.019	0.002	0.035[a]	0.061[a]	0.014	0.015	0.016	0.013	0.012
BAN11p	0.010	0.011	0.001	0.005	0.009	0.010	0.002	0.011	0.010	0.011
BAN38p	0.008	0.011	0.001	0.004	0.008	0.009	0.002	0.011	0.011	0.010

Table 15 continued.

	INTO								
		in the pasture						near BAN11p	
FROM	BAN17t (n = 1)	BAN09p (n = 10)	BAN19t (n = 2)	BAN34t (n = 2)	BAN35t (n = 1)	BAN36t (n = 1)	BAPp (n = 21)	BAN11p (n = 2)	BAN38p (n = 2)
BAN37t	0.010	0.006	0.010	0.012	0.010	0.011	0.002	0.011	0.011
BAN32p	0.008	0.006	0.011	0.010	0.010	0.010	0.001	0.010	0.011
BAN02p	0.011	0.034[a]	0.022	0.032[a]	0.015	0.012	0.003	0.019	0.019
BAN12t	0.010	0.034[a]	0.020	0.014	0.011	0.010	0.002	0.027	0.022
BAN14t	0.010	0.005	0.011	0.011	0.010	0.011	0.001	0.012	0.011
BAN23t	0.008	0.007	0.011	0.012	0.010	0.008	0.002	0.011	0.010
BAN04p	0.012	0.031	0.032[a]	0.016	0.013	0.012	0.003	0.016	0.019
BAN15t	0.009	0.005	0.011	0.010	0.011	0.009	0.001	0.011	0.010
BAN24t	0.010	0.005	0.009	0.010	0.008	0.010	0.001	0.010	0.009
BAN07p	0.009	0.005	0.011	0.012	0.010	0.009	0.001	0.010	0.010
BAN17t	**0.825**[a]	0.007	0.012	0.012	0.012	0.010	0.002	0.011	0.010
BAN09p	0.012	**0.738**[a]	0.014	0.012	0.011	0.010	0.002	0.013	0.013
BAN19t	0.011	0.006	**0.755**[a]	0.013	0.012	0.011	0.001	0.010	0.010
BAN34t	0.010	0.006	0.011	**0.762**[a]	0.010	0.011	0.001	0.011	0.010
BAN35t	0.008	0.006	0.011	0.012	**0.806**[a]	0.010	0.001	0.011	0.010
BAN36t	0.009	0.008	0.011	0.012	0.011	**0.814**[a]	0.002	0.011	0.011
BAPp	0.011	0.078[a]	0.019	0.020	0.010	0.010	**0.970**	0.024	0.023
BAN11p	0.008	0.006	0.010	0.011	0.011	0.010	0.001	**0.761**[a]	0.011
BAN38p	0.009	0.007	0.009	0.010	0.011	0.010	0.002	0.011	**0.768**[a]

Source groups (FROM are listed in the left-hand column, receiving groups (INTO) are listed across the top row. Bold values are migration rates >0.1. All values were consistent across ten runs.
[a]Standard deviation was 0.051–0.115. All other standard deviations were <0.05.

TABLE 16. Analysis of molecular variance (AMOVA) results for different hierarchical models. Genetic variance is partitioned among (A) groups identified by GENELAND version 3.1.4 or (B) and (C) groups identified via multiple methods.

Hierarchical models	Source of variation	% total variance	*P*
(A) Groups identified by Geneland analysis A (clusters O and X)			
	Among groups	6.28	<0.00001
	Among sites	8.93	<0.00001
	Within sites	84.79	<0.00001
(B) Nine groups detected via multiple methods (Cameron, HAR01s, HAR02s, Hill, Liberty, MEX04s, other Mexico sites, Terrell & Val Verde, and cluster X)			
	Among groups	8.15	<0.00001
	Among sites	7.53	<0.00001
	Within sites	84.32	<0.00001
(C) Nineteen groups detected via multiple methods (groups Cameron; HAR01s; HAR02s; Hill; Liberty; MEX04s; other Mexico sites; Terrell & Val Verde; Aransas; Austin & Colorado; Bandera; Bastrop, Lee, & Travis; Edwards; Ft. Bend; Guadalupe & Hays; Kenedy; Lavaca; Leon; and east cluster X)			
	Among groups	5.57	<0.00001
	Among sites	6.21	<0.00001
	Within sites	88.22	<0.00001

TABLE 17. Average uncorrected pairwise divergences among species (below diagonal, shaded) and estimated divergence dates among and within species (mya, along and above diagonal).

	B. melanochlorus	*B. valliceps*	*B. nebulifer*
B. melanochlorus	—	3.833	7.727 – 7.493
B. valliceps	12.600 %	—	7.071 – 6.867
B. nebulifer	24.910 %	22.963 %	0.574 – 0.115

mya = million years ago

FIG 1. (a) Global range of *Bufo nebulifer* (data source: IUCN [International Union for Conservation of Nature], Conservation International & NatureServe; Hammerson & Canseco-Márquez 2004). Inset is Fig. 1(b). (b) Sites sampled in Texas and Tamaulipas (see also Tables 4–5). The nine clusters recovered in genetic analyses are indicated by dashed lines and are labeled.

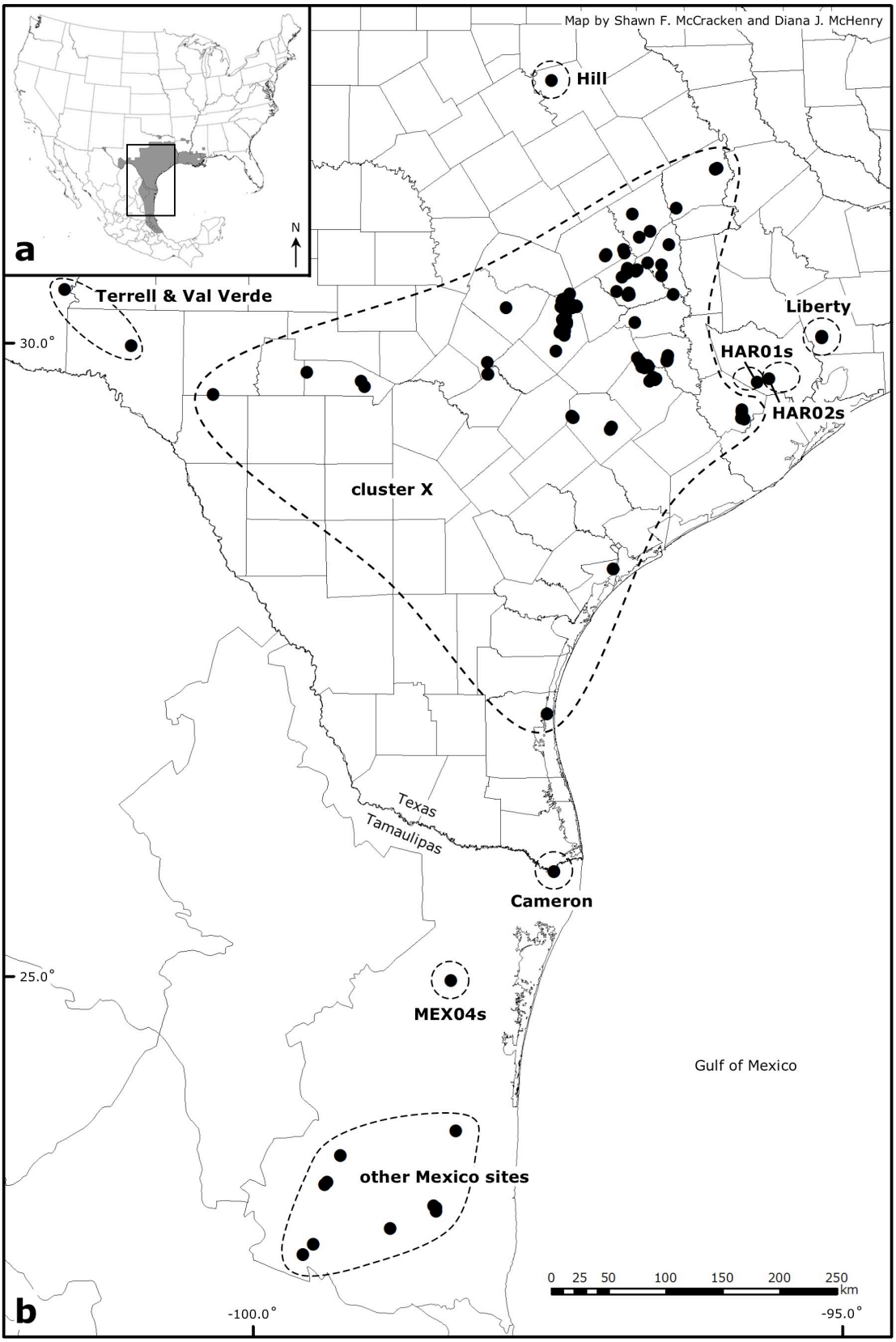

Map by Shawn F. McCracken and Diana J. McHenry
a
N
Hill
Terrell & Val Verde
30.0°
Liberty
HAR01s
HAR02s
cluster X
Texas
Tamaulipas
Cameron
25.0°
MEX04s
Gulf of Mexico
other Mexico sites
b
0 25 50 100 150 200 250
km
-100.0°
-95.0°

FIG. 2. Maximum likelihood phylogram of 12 unique mtDNA haplotypes (148 individuals) rooted with *Bufo melanochlorus* (MF04527 mel). Haplotypes occurring in multiple individuals have four letter designations followed by sample size; haplotypes occurring in only one individual are denoted by MF# followed by an abbreviation of the specific epithet (e.g., mel = *B*. *melanochlorus*). MP bootstraps, ML bootstraps, and Bayesian posterior probabilities are shown above branches. Eleven haplotypes comprised the ingroup: one *B*. *valliceps* and 10 *B*. *nebulifer*. One *B*. *valliceps* (MF06336 val) was included in the ingroup.

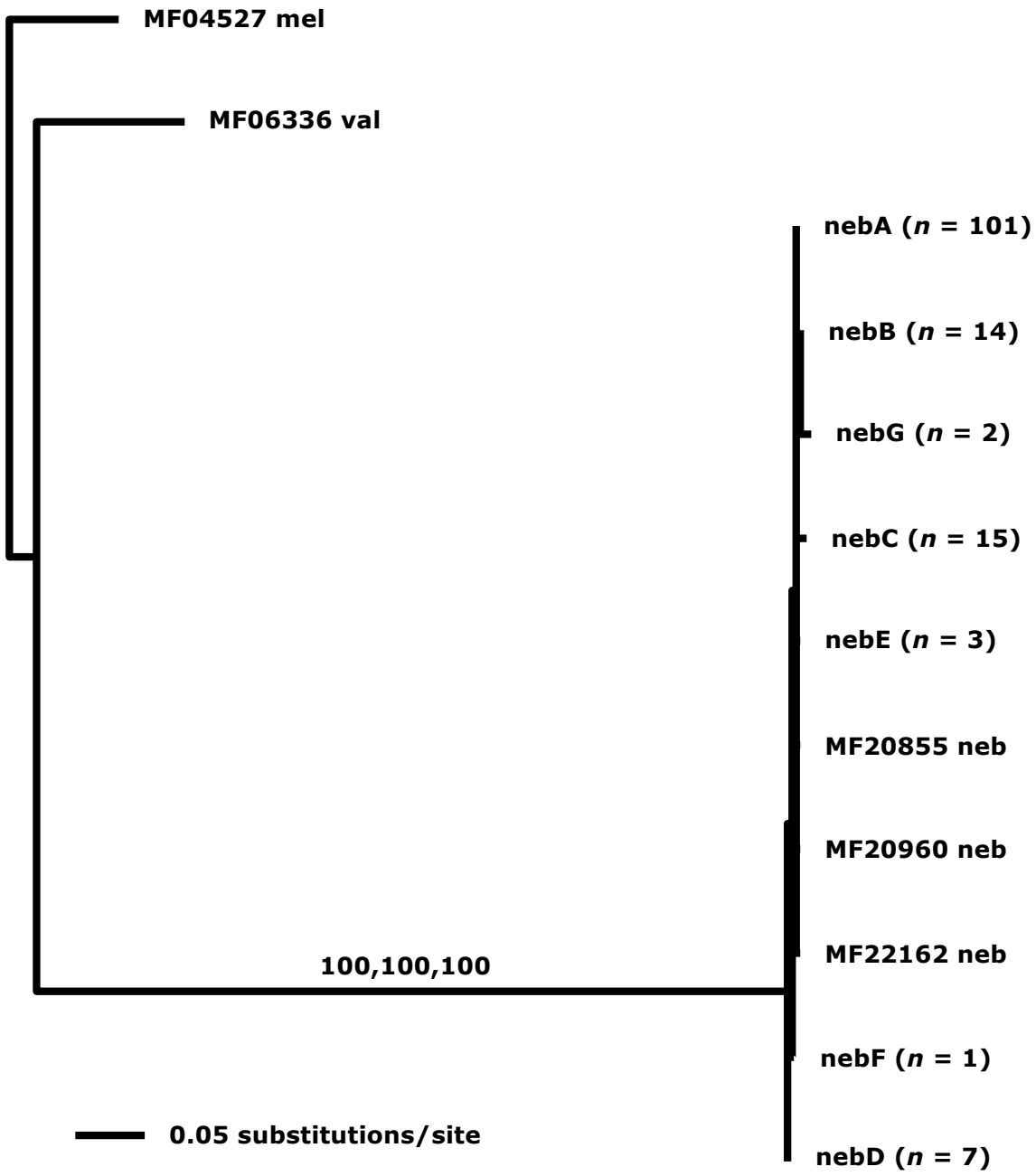
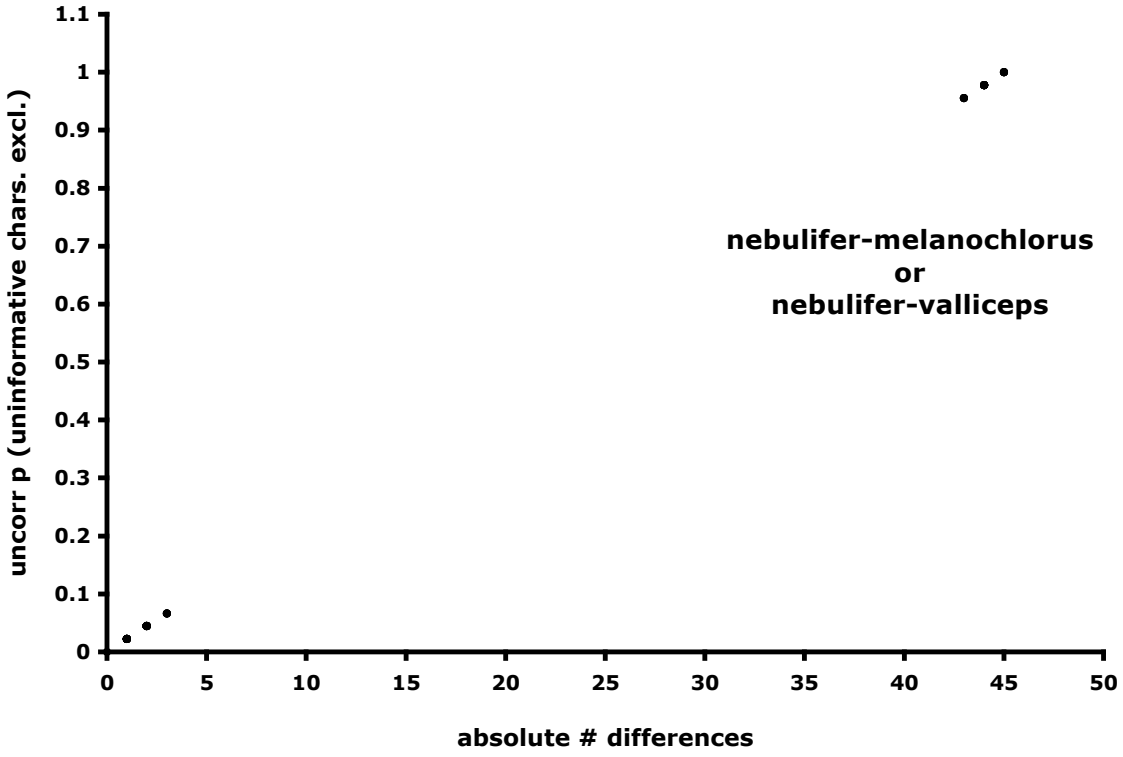

MF04527 mel
MF06336 val
nebA (n = 101)
nebB (n = 14)
nebG (n = 2)
nebC (n = 15)
nebE (n = 3)
MF20855 neb
MF20960 neb
MF22162 neb
100,100,100
nebF (n = 1)
nebD (n = 7)
0.05 substitutions/site

FIG. 3. (a) Uncorrected pairwise distance (after excluding uninformative characters) plotted against absolute number of differences among *Bufo nebulifer*, *B*. *melanochlorus*, and *B*. *valliceps*. Pairwise comparisons of nebulifer-melanochlorus and nebulifer-valliceps are indicated by the grey circle. Data points not enclosed in a grey circle are comparisons within nebulifer and between melanochlorus and valliceps. Saturation was not observed. (b) Uncorrected pairwise distance of transitions (black squares) and transversions (grey circles) plotted against absolute distance. Saturation of transitions or of transversions was not observed.


uncorr p (uninformative chars. excl.)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
absolute # differences
nebulifer-melanochlorus
or
nebulifer-valliceps

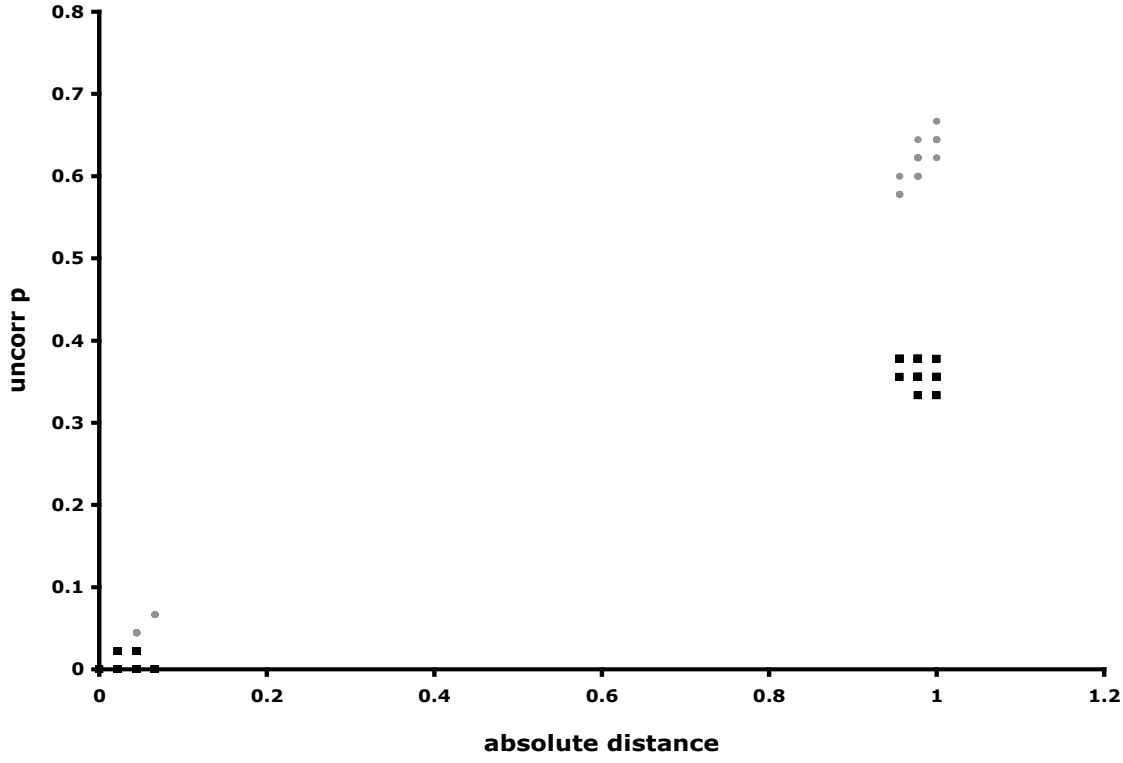

uncorr p
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
1.2
absolute distance

FIG. 4. Statistical parsimony network of 10 mtDNA haplotypes in 146 *Bufo nebulifer*. Circle size is proportional to number of individuals: nebA (*n* = 101), nebB (*n* = 14), nebC (*n* = 15), nebD (*n* = 7), nebE (*n* = 3), nebF (*n* = 1), nebG (*n* = 2), MF20855 (*n* = 1), MF20960 (*n* = 1), and MF22162 (*n* = 1). Each line represents a single mutation; small filled circles represent nonsampled or extinct haplotypes.

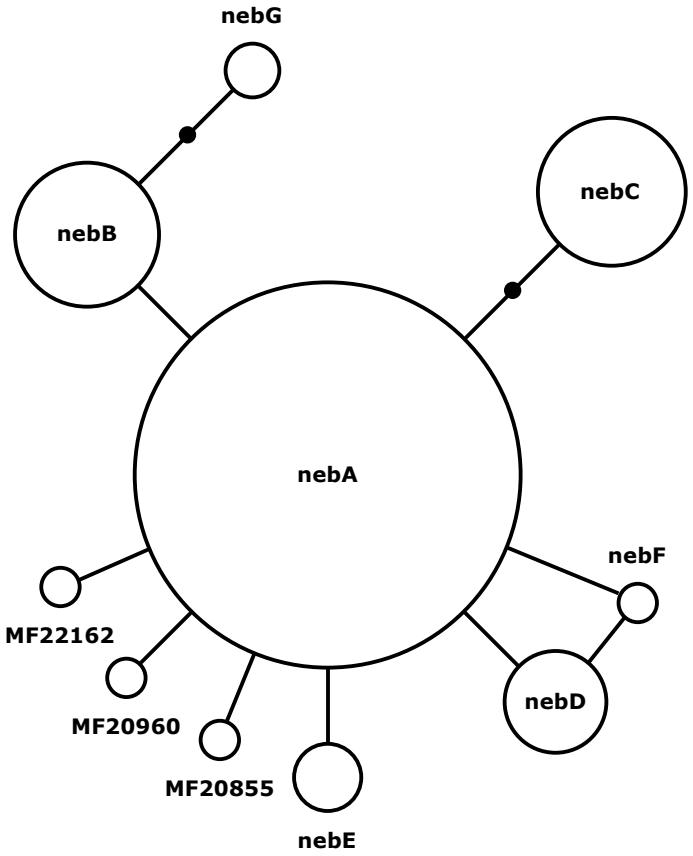
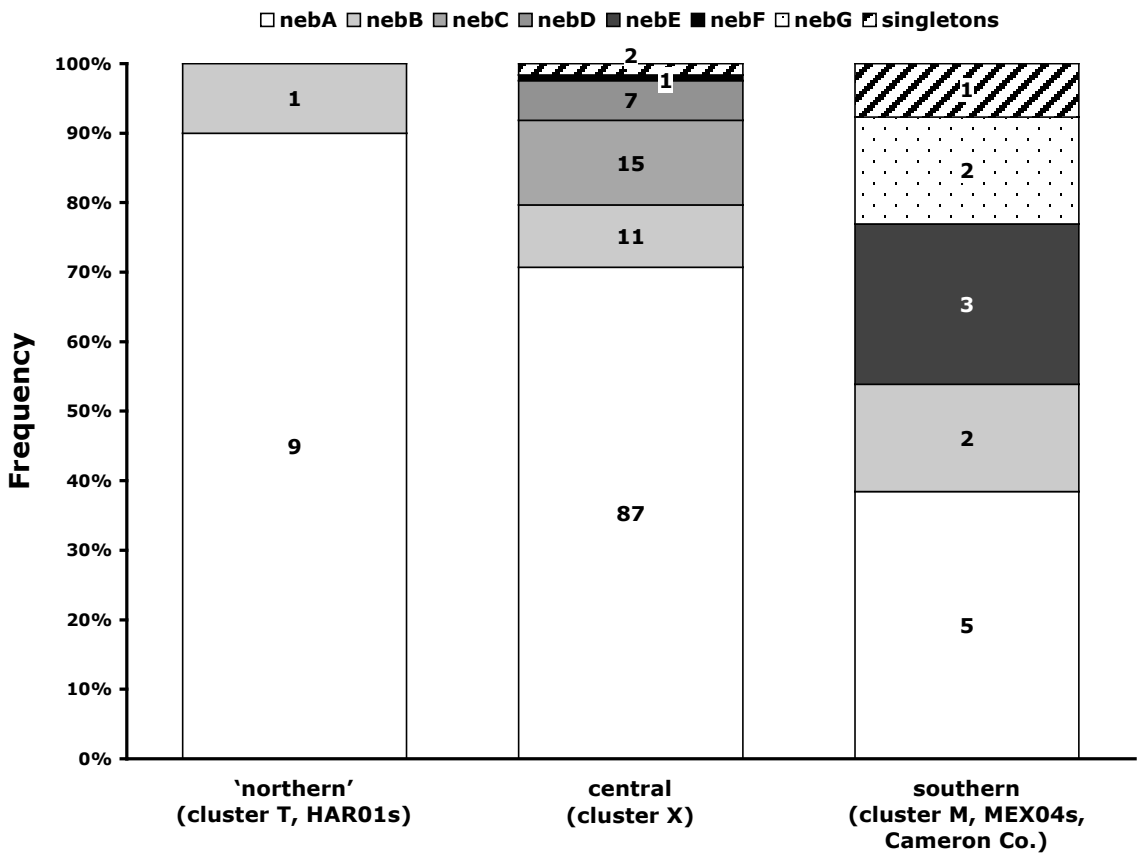

nebG
nebB
nebC
nebA
nebF
MF22162
MF20960
MF20855
nebE
nebD

FIG. 5. Frequency of *Bufo nebulifer* mtDNA haplotypes in three main geographic regions: 'northern' (cluster T [Terrell, Val Verde, Hill, and Liberty counties, HAR01s, and HAR02s] and HAR01s), central (cluster X), and southern (cluster M, MEX04s, and Cameron Co.). Three unique haplotypes are categorized together as 'singletons': MF20855, MF20960, and MF22162.


nebA
nebB
nebC
nebD
nebE
nebF
nebG
singletons
Frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
9
2
1
7
15
11
87
1
2
3
2
5
'northern'
(cluster T, HAR01s)
central
(cluster X)
southern
(cluster M, MEX04s,
Cameron Co.)

Chapter 4

MEASURING NATURAL LEVELS OF INTERSPECIFIC ADMIXTURE IN AN ENDANGERED TOAD

ABSTRACT: To effectively protect endangered species, natural levels of interspecific hybridization, or admixture, must be estimated, especially in cases where anthropogenic changes to the environment may broaden contact between the species. Here, I investigate the baseline levels of admixture in *Bufo houstonensis* using mitochondrial sequence data and microsatellite loci. Admixture between *B. houstonensis* and two sympatric species — *B. nebulifer* and *B. woodhousii* — was detected. Phenotype-based assessments of admixture appear to be temporally stable, but they underestimate true levels of admixture. *Bufo nebulifer* × *B. houstonensis* F_1 hybrids can be fertile and backcross to *B. nebulifer*; *B. nebulifer* × *B. houstonensis* matings may result in fertile offspring more frequently than previously thought. Admixed individuals with *B. houstonensis* or *B. woodhousii* maternal lineages can backcross to *B. houstonensis*. Phenotypically aberrant individuals were not always F_1 hybrids, and F_1 hybrids were not always phenotypically aberrant. With continued habitat alteration and rising temperatures, habitat isolation and offset breeding season have already partially broken down and may deteriorate further; consequently, opportunities for hybridization events will increase. Selection against hybrids at the tadpole stage did not occur among *B. houstonensis* and *B. nebulifer* individuals. All these factors may lead to higher levels of gamete wastage in *B.*

houstonensis, an already critically endangered species.

KEY WORDS: *Bufo houstonensis*, hybridization, endangered species, interspecific admixture

INTRODUCTION

Detecting hybridization, or admixture, between taxa is key for conservation management of endangered species. Interspecific admixture threatens endangered or rare taxa via gamete wastage, population-wide lowered fitness due to the presence of less fit hybrids, and extinction through introgression or through competition with heterotic hybrids. Hybridization is a natural occurrence for many taxa —from sunflowers (Carney et al. 2000) to sea turtles (Lara-Ruiz et al. 2006) to canids (Miller et al. 2003). However, admixture resulting from human-caused changes in the environment has also been reported (Levin et al. 1996, Allendorf et al. 2001, Fitzpatrick & Shaffer 2007a, Pasachnik et al. 2009). The introduced barred tiger salamander (*Ambystoma tigrinum mavortium*) hybridizes with the California tiger salamander (*Ambystoma californiense*) and benefits from human modification of its natural breeding habitat, ephemeral pools, into perennial pools (Fitzpatrick & Shaffer 2004, 2007a, 2007b). And in two Honduran iguana species (*Ctenosaura bakeri* and *C. similis*), contact between them appears to be increased where anthropogenic change has occurred, around homes where both species inhabit cinder block walls and where both species are fed daily (Pasachnik et al. 2009). To understand

the implications of human-induced hybridization and to manage endangered taxa, levels of natural admixture must be measured.

Admixture among toad species in the family Bufonidae is well known to occur naturally (Blair 1972), but a few recent studies have implicated anthropogenic causes in some hybridization events (Gergus et al. 1999, Dixon 2000, Vogel & Johnson 2008). The Houston toad, *Bufo houstonensis* (= *Anaxyrus houstonensis*, Frost et al. 2006a) is endemic to southeast-central Texas and is listed as endangered at the State and Federal levels (Gottschalk 1970, Potter et al. 1984, Campbell 2003). Natural hybrids with *B. nebulifer* (= *Incilius nebulifer*, Mulcahy & Mendelson 2000, Frost et al. 2006a, Frost et al. 2006b, Frost et al. 2009) and with *B. woodhousii* (= *Anaxyrus woodhousii*, Frost et al. 2006a) have been found (Brown 1971b, Hillis et al. 1984), and laboratory crosses with other bufonid species result in viable or fertile offspring (Blair 1959, 1963, 1972). Brown & Thomas (1982) assert that hybridization is not a cause of the decline in *B. houstonensis*, because few natural hybrids have been found and they were localized in time and space. However, natural hybridization is recognized to have the potential to contribute towards extinction of *B. houstonensis* (Brown 1971b, Brown & Thomas 1982). This potential is likely exacerbated by anthropogenic changes in the environment, such as habitat alteration or destruction, especially deforestation and water impoundments. Impounding water directly impacts *B. houstonensis* through the actual construction, loss of natural ephemeral ponds, and concentration of livestock around ponds (Seal 1994). Further, permanent impoundments attract *B. houstonensis*, though they prefer ephemeral ponds, but they also attract *B. nebulifer* and *B. woodhousii* (Seal 1994) which increases

the opportunities for hybridization. *Bufo houstonensis* prefer canopied habitats and deforestation directly impacts the toad through loss and modification of habitat, but also raises the likelihood for hybridization by creating habitats preferable to *B. nebulifer* and *B. woodhousii* (Kennedy 1961, Brown 1971b, Thomas & Potter 1975, Potter et al. 1984, Mendelson 2005, Sullivan 2005).

Almost certainly, anthropogenic changes to the environment have already increased the likelihood of interspecific hybridization events. Natural *Bufo houstonensis* × *B. woodhousii* hybrids have previously been found only at human-made ponds, typically near recent construction or near forest/pasture edges (Brown 1967, 1971b, Hillis et al. 1984). At one site in Bastrop County, Texas, *B. houstonensis* × *B. woodhousii* hybrids and both parental species were found in the mid-1960s after construction in the area, but by 1979 no *B. houstonensis* and only *B. woodhousii* were found (Hillis et al. 1984); this example demonstrates how hybridization might be indicative of a transition step towards local extirpation of *B. houstonensis*. Natural *B. houstonensis* × *B. nebulifer* hybrids have previously been found at ponds near roadways but also at relatively undisturbed forested sites (Brown 1967, 1971b, Hillis et al. 1984), so it is possible that anthropogenic changes have yet to influence hybridization between *B. houstonensis* and *B. nebulifer*. Levels of admixture are still unknown (i.e., are they F_1 hybrids, F_2 hybrids, and/or backcrosses?), and measuring baseline levels of natural hybridization is necessary to assess the full impact of current and future anthropogenic habitat modification and consequent changes in interspecific admixture. These baseline levels were investigated in the present study using mitochondrial sequence data and nuclear microsatellite loci. In

addition to determining how much admixture exists in *B*. *houstonensis*, the types of hybrid classes, the temporal and geographic limits of admixture, and selection against hybrids at different life-history stages were also examined.

MATERIALS AND METHODS

Sampling. *Bufo houstonensis* and *B*. *woodhousii* individuals were sampled as described in Chapter 2, and *B*. *nebulifer* individuals were sampled as described in Chapter 3 (see also Appendix A). Tissues were sampled and stored as described in Chapter 2. Sampling was performed under permits and approvals described in Chapters 2 and 3.

At the time of sampling, individuals with morphological or advertisement call characteristics intermediate between/among species were termed putative hybrids. Typically, *Bufo houstonensis* (Fig. 1a) have thickened cranial crests, elongate parotoid glands closest together at midpoint, and dark vocal sacs in males (Sanders 1953, Thomas & Potter 1975, Conant & Collins 1998, Dixon 2000); parotoid glands are sometimes in contact with preparotoid and/or postorbital cranial crests (Thomas & Potter 1975). *Bufo nebulifer* (Fig. 1b) have prominent, but not thickened, cranial crests bordering a wide valley between the eyes, tear-shaped or triangular parotoid glands, and yellowish vocal sacs in males (Conant & Collins 1998, Dixon 2000). While both have trilled advertisement calls, *B*. *houstonensis* calls are a higher frequency (2300 vs. 1250-1800 cycle per sec) and longer duration (3.8-11.2 vs. 1.9-5.0 sec) than *B*. *nebulifer* calls (Blair

1956a). Finally, *B*. *woodhousii* have prominent cranial crests, elongate parotoid glands closest together anteriorly, and dark vocal sacs in males (Conant & Collins 1998, Dixon 2000); the advertisement call of *B*. *woodhousii* is short (0.8-2.7 sec) and not trilled (Blair 1956a).

DNA methods. DNA was isolated as described in Chapter 2. Control region (D-loop) mitochondrial (mtDNA) haplotype sequences were obtained as described in Chapters 2 and 3. Data for 11 microsatellite loci (BBR34-2, BBR36, BBR86, BBR281, BC52.03, BC52.10, BC52.12, bco15, BM224other, IHHH, and IYY) were obtained as described in Chapters 2 and 3 (see also citations therein).

Genetic assignment analyses. After microsatellite loci were scored and sequences were known, some individuals were termed predefined hybrids. These included the putative hybrids (described above), individuals with mismatches between phenotype and mtDNA (e.g., phenotype was *Bufo houstonensis* and mtDNA was a *B*. *nebulifer* haplotype), individuals with mismatches between phenotype and diagnostic alleles, individuals with mismatches between mtDNA and diagnostic alleles, or individuals with mismatches among diagnostic alleles. Five loci consistently amplified in all three parental species (BBR36, BC52.10, bco15, BM224other), one in only *B*. *houstonensis* (IYY), one in only *B*. *nebulifer* (BBR86), and five in *B*. *houstonensis* and *B*. *woodhousii* (BBR34-2, BBR281, BC52.03, BC52.12, IHHH). Loci BBR281 and BC52.12 were tested in, and amplified in, only a very few *B*. *nebulifer*. For locus bco15, *B*. *nebulifer* individuals were fixed for a private allele. Diagnostic alleles were categorized as Nearctic (amplified in *B*. *houstonensis* and/or *B*. *woodhousii*) or *B*.

nebulifer. Individuals with both Nearctic and *B*. *nebulifer* alleles (i.e., mismatch between diagnostic alleles) were deemed predefined hybrids. Failure to amplify was not considered diagnostic; for example, failure to amplify for locus BBR86 was not indicative of a Nearctic allele.

STRUCTURE version 2.1 (Pritchard et al. 2000) was used to infer the number of clusters (*K*), or populations, in the dataset and to assign the proportions of an individual's genotype to the parental species. Preliminary analyses indicated that although there were three species (*Bufo houstonensis*, *B*. *nebulifer*, and *B*. *woodhousii*) in my study system, there were more than three clusters in the dataset. Bayesian analyses (both correlated and independent allele frequencies) in STRUCTURE were performed as described in Chapter 2 for $K = 1$ to $K = 5$ on the entire dataset ($n = 1112$).

NEWHYBRIDS version 1.1 (Anderson & Thompson 2002) was used to assign individuals to one of the following genotype classes: parental species (*Bufo houstonensis*, *B*. *nebulifer*, or *B*. *woodhousii*), cross between parentals (F_1), cross between F_1s (F_2), or backcross between a parental and an F_1 (BX). Because there are three parental species, any of which can hybridize (Thornton 1955, Blair 1956b, Kennedy 1961, Brown 1971a, 1971b, Brown & Brownell 1971, Hillis et al. 1984), pairwise sub-analyses had to be performed for each analysis: *B*. *houstonensis* with *B*. *nebulifer*, *B*. *houstonensis* with *B*. *woodhousii*, and *B*. *nebulifer* with *B*. *woodhousii*. Multiple analyses were performed to find all possible admixed individuals in the dataset. In the first analysis (analysis A, Table 1), predefined hybrids were excluded. Predefined hybrids were included in analysis B. Any individual initially identified as a parental species but assigned to a hybrid class in

either analysis was termed a 'possible' hybrid. Both predefined and 'possible' hybrids were excluded from the dataset in analysis C. Again, any individual initially identified as a parental species but assigned to a hybrid class in this analysis was termed a 'possible' hybrid. Predefined and 'possible' hybrids were included in a final analysis (analysis D). For each run, the following parameters were used: 100,000 burn-in sweeps, 1,000,000 sweeps, Jeffreys-like priors, and six genealogical classes at default genotype frequencies. To assign an individual to a genotype class, a posterior probability of 0.5 was used as the threshold.

Simulated assignment analyses. The ability to detect admixed individuals was assessed by simulation. Parental, F_1, F_2, and BX genotypes were simulated in HYBRIDLAB version 1.0 (Nielsen et al. 2006). Experimental parental genotypes with $Q \geq 0.98$ (Q = the estimated membership coefficient for an individual for that species estimated in STRUCTURE) were used to create 20 simulated parental genotypes per species. These were used to simulate F_1 genotypes which were then used to simulate F_2 genotypes; BX genotypes were created by crossing simulated parentals with simulated F_1s. Table 2 shows all the types of hybrid crosses that were simulated; 20 genotypes per cross were generated. Bayesian analysis in STRUCTURE was performed on the simulated dataset as described above, but only for $K = 3$ ($n = 360$). Three subsets were also analysed in NEWHYBRIDS: 1) simulated *Bufo houstonensis*, simulated *B*. *nebulifer*, and simulated hybrids (F_1s, F_2, and BXs); 2) simulated *B*. *houstonensis*, simulated *B*. *woodhousii*, and simulated hybrids; and 3) simulated *B*. *nebulifer*, simulated *B*. *woodhousii*, and simulated hybrids. These subsets were analysed using the z option for

the simulated parental individuals.

Genetic diversity analyses. Once an admixture threshold was determined, admixed individuals and predefined hybrids were excluded from the dataset to perform the following analyses. For the three parental species, allele frequencies and number of private alleles (A_p) were estimated using FSTAT version 2.9.3 (Goudet 2001). Exact tests for Hardy-Weinberg equilibrium (HWE) were performed with 1,000,000 Markov chain steps and 100,000 dememorisation steps in ARLEQUIN version 3.11 (Excoffier et al. 2005). Tests for linkage disequilibrium (LDE) among loci, within or among samples, were performed in FSTAT version 2.9.3 with 3300 permutations. Significance, of HWE and of LDE, was determined after sequential Bonferroni correction with $\alpha = 0.05$ (Rice 1989). Differences in allele frequencies among the three parental species were assessed by computing pairwise F_{ST} values in ARLEQUIN version 3.11 (Excoffier et al. 2005) with 10,000 permutations and a significance value of 0.05.

Hybridity. Kruskal-Wallis one-way ANOVAs were used to assess differences in hybridity among life-history stages. Hybridity is a measure of genetic intermediateness and was calculated for each individual with the formula $h_i = 0.5 - |0.5 - Q|$ (sensu Carney et al. 2000, Gow et al. 2007), where Q = the estimated membership coefficient for an individual for that species estimated in STRUCTURE. Two analyses were performed because two species are sympatric with and hybridize with *Bufo houstonensis*: 1) individuals with phenotypes of *B. houstonensis* or *B. nebulifer* (n = 1037) and 2) individuals with phenotypes of *B. houstonensis* or *B. woodhousii* (n = 488). Mean hybridity was calculated for three life-history stages in both analyses: tadpole, juvenile,

and adult. Individuals whose life-history stage was egg or indeterminate and whose phenotype was putative hybrid were excluded from analyses. If a significant result was found, then a nonparametric multiple comparisons with unequal samples sizes test was performed.

RESULTS

In total, 1112 individuals were sampled: 439 *Bufo houstonensis*, 600 *B*. *nebulifer*, and 26 *B*. *woodhousii* (six more *B*. *woodhousii* than in Chapter 2 were included here and all were from Hill County, Texas; Appendix A). Nine were putative hybrids (two examples are shown in Fig. 2). Forty-seven individuals (which included the nine putative hybrids) were termed predefined hybrids because of mismatches (see Materials and Methods).

Genetic assignment analyses. According to the ad hoc measures of Evanno et al. (2005), the most likely number of clusters was 2. However, from $K = 2$ to $K = 4$, all *Bufo woodhousii* individuals were assigned to *B*. *houstonensis* clusters in some runs and sometimes to *B*. *nebulifer* clusters in other runs within the same K value. Only at $K = 5$ were *B*. *woodhousii* individuals assigned to a unique cluster. At $K = 5$, two other clusters were *B*. *houstonensis* clusters (and gave similar results to those reported in Chapter 2), and the remaining two were *B*. *nebulifer* clusters. To assess levels of admixture, Q values for the two *B*. *houstonensis* clusters were summed to create one *B*. *houstonensis* cluster and Q values for the two *B*. *nebulifer* clusters were summed to create one *B*. *nebulifer*

cluster, such that $K = 3$. Results from independent and correlated allele frequencies were similar, so only the results from the correlated allele frequencies analysis are presented here. Results from STRUCTURE analysis are summarized in Table 3 and Fig. 3. At a Q value threshold of 0.9, if $Q < 0.9$ then an individual was considered admixed; similarly at the 0.85 threshold, if $Q < 0.85$ then an individual was considered admixed. At the Q value threshold of 0.9, 91 % of parentals were assigned to the species to which it was identified pre-analysis, and at a 0.85 threshold, 93.6 % were. Of 439 *B*. *houstonensis*, 43 (9.8 %) were admixed at 0.9 and 32 (7.4 %) at 0.85. Twenty-one (44.7 %) of the 47 predefined hybrids were admixed: 11 (52.4 %) had Q values higher than 0.1 for *B*. *houstonensis* and *B*. *nebulifer* clusters, 7 (33.3 %) for *B*. *houstonensis* and *B*. *woodhousii* clusters, 1 (4.8 %) for *B*. *nebulifer* and *B*. *woodhousii* clusters, and 2 (9.5 %) for all three parental clusters (Tables 3 and 4). The only county where admixture in *B*. *houstonensis*-phenotype individuals was not present was Colorado County. *Bufo houstonensis* were sampled most intensively in the populations in Bastrop County (populations BAPp, BAS06p, N, S_1, and S_2; see Chapter 2). In adult toads, admixture was most frequent in population BAS06p: 10.3 % ($n = 39$), 11.8 % ($n = 17$), 9.3 % ($n = 182$), 9.1 % ($n = 66$), and 5.2 % ($n = 58$), respectively. Table 5 shows the number and percentage of admixed individuals per year for populations of *B*. *houstonensis* in Bastrop County and for *B*. *nebulifer* from the same areas (although *B*. *nebulifer* was only one population in Bastrop County, see Chapter 3). For *B*. *nebulifer*, admixture was highest in area S_2 (21.2 %) and lowest in area N (3.4 %). Among years, percentage of admixed individuals varied for both species. Fig. 4 shows the percentage of admixed adults in population/area N for both

species; more *B*. *houstonensis* individuals were admixed than *B*. *nebulifer* (9.3 % vs. 3.4 %). In contrast, at population/area S_2, more *B*. *nebulifer* individuals were admixed than *B*. *houstonensis* (21.2 % vs. 5.2 %). Fig. 5 shows total number of adults sampled and percentage of adults that were admixed by population (BAPp, BAS06p, N, S_1, and S_2) for *B*. *houstonensis* and for *B*. *nebulifer*. Admixture was detected for sample sizes as low as 17; percentage of admixed individuals did not correlate total number of individuals sampled.

Results from NEWHYBRIDS analysis D are summarized in Table 3 and Fig. 3. All individuals that were assigned to a hybrid class were assigned to the F_2 class. The chances of all hybrid individuals being F_2s are very low, thus, distinct hybrid classes were not informative. Consequently, the categories used for assignments were parental *Bufo houstonensis*, parental *B*. *nebulifer*, parental *B*. *woodhousii*, and admixed. For all parental species, 1041 (97.7 %) individuals were assigned to the species to which it was identified pre-analysis; 425 (96.8 %) individuals identified as *B*. *houstonensis* were assigned to *B*. *houstonensis*. Fifteen (31.9 %) of the 47 predefined hybrids were admixed (Tables 3 and 4).

Of the 28 predefined hybrids with *Bufo woodhousii* mtDNA haplotypes, 19 (67.9 %) were assigned to *B*. *houstonensis* at *Q* threshold = 0.9 and were also assigned to *B*. *houstonensis* in NEWHYBRIDS analysis D (Table 4). Twenty-one (75 %) were assigned to *B*. *houstonensis* at $Q \geq 0.87$ (all 21 were assigned to *B*. *houstonensis* in NEWHYBRIDS analysis D). The remaining 7 had *Q* values for the *B*. *houstonensis* cluster ranging from 0.37 to 0.72 (mean = 0.6; 2 were assigned to *B*. *houstonensis* and 5 were admixed in

NEWHYBRIDS analysis D). Of the 10 predefined hybrids with *B*. *nebulifer* haplotypes, none were assigned to *B*. *houstonensis* (Q = 0.006-0.231, mean = 0.071; none were assigned to *B*. *houstonensis* in NEWHYBRIDS analysis D), but 6 (60 %) were assigned to *B*. *nebulifer* (all 6 were assigned to *B*. *nebulifer* in NEWHYBRIDS analysis D). Of the 6 predefined hybrids with *B*. *houstonensis* haplotypes, 1 (16.7 %) was assigned to *B*. *houstonensis* (this individual was also assigned to *B. houstonensis* in NEWHYBRIDS analysis D) and the remaining were admixed in both analyses ($Q_{B.\ houstonensis}$ ranged 0.241-0.729).

Simulated assignment analyses. Results from independent and correlated allele frequencies analyses of the simulated dataset in STRUCTURE were similar, so only the results from the correlated allele frequencies analysis are presented here (Table 6 and Fig. 6). At a Q value threshold of 0.9, 56 (93.3 %) of parentals were correctly assigned and 292 (97.3 %) of hybrids were assigned as admixed. At a Q value threshold of 0.85, 58 (96.7 %) parentals were correctly assigned and 278 (92.7 %) hybrids were assigned as admixed. In NEWHYBRIDS, 60 (100 %) parentals were correctly assigned and 219 (73 %) hybrids were assigned as admixed. Using either 0.9 or 0.85 as a Q value threshold identifies the majority of simulated hybrids, so at these values of F_{ST} (0.15093-0.24823, see below) among parentals, 11 microsatellite loci appear to be sufficient to detect admixture. Assignments in STRUCTURE were wrong 4.7 % of the time at 0.9 and 5.3 % at 0.85. Because fewer mistakes occurred and more hybrids were identified at Q value threshold = 0.9, this appears to be the more appropriate threshold for this dataset.

Genetic diversity analyses. Twelve *Bufo houstonensis* mtDNA haplotypes, 7 *B*.

nebulifer, and 6 *B*. *woodhousii* were found (see Chapter 2 Fig. 2 for a phylogenetic tree of Nearctic species and Chapter 3 Fig. 2 for a phylogenetic tree of *B*. *nebulifer*). Characteristics of genetic diversity are presented in Table 7. Number of alleles per species ranged 66-146, while number of private alleles ranged 25-88. After sequential Bonferroni correction, no loci significantly deviated from HWE. Loci BC52.03 and IHHH were determined to be in LDE among all three species ($P = 0.00030$, $\alpha = 0.000303$) and in *B*. *houstonensis* ($P = 0.00030$, $\alpha = 0.000303$). Loci BBR36 and BC52.10 were in LDE in *B*. *houstonensis* ($P = 0.00030$, $\alpha = 0.000303$). Pairwise F_{ST} values were calculated among species (Table 8): the lowest was between *B*. *houstonensis* and *B*. *nebulifer* (0.15093), while the highest was between *B*. *nebulifer* and *B*. *woodhousii* (0.24823).

Hybridity. Mean hybridity (Fig. 7) was not significantly different across life-history stages (tadpole, juvenile, and adult) for *Bufo houstonensis* and *B*. *nebulifer* ($H = 3.78$, $P_{0.05,2} = 0.1511$). However, it was significantly different for *B*. *houstonensis* and *B*. *woodhousii* ($H = 17.78$, $P_{0.05,2} = 0.0001$); mean hybridity decreases from the tadpole stage to the juvenile stage ($Q = 4.212$, $Q_{critical} = 2.394$) and to the adult stage ($Q = 3.119$, $Q_{critical} = 2.394$).

DISCUSSION

Only recently has the phenomenon of vertebrate interspecific hybridization begun to be appreciated and investigated (Rhymer & Simberloff 1996, Dowling & Secor 1997,

Allendorf et al. 2001, Seehausen 2004, Mallet 2005). Naturally occurring hybrids are now known from a wide range of vertebrates (Rhymer & Simberloff 1996, Dowling & Secor 1997, Mallet 2005), and in some cases, introgression has been demonstrated to occur (e.g., Bertier et al. 2006, Barilani et al. 2007, Yamazaki et al. 2008, Kidd et al. 2009). For rare or endangered taxa, hybridization and/or introgression can contribute to further declines (Rhymer & Simberloff 1996, Allendorf et al. 2001). Previous workers (e.g., Brown 1967, 1971b, Brown & Thomas 1982, Hillis et al. 1984) have argued that hybridization should not be emphasized as a cause of decline in the endangered *Bufo houstonensis* and that there is no evidence of widespread hybridization, backcrossing, or introgression.

While there are likely far more important causes of decline in this species — habitat loss/fragmentation/alteration, climate change, disease — there is now evidence of higher levels of admixture than previous studies suggested. Previous studies may have underestimated the level of admixture (hybridization, backcrossing, and/or introgression) for the examined populations; alternatively, *Bufo houstonensis* may have experienced an increase in hybridization events since those studies. In either case, the results of the present investigation cannot be dismissed. Also key is that the identification of an individual as being 'hybrid', or as 'pure', based on morphological or behavioral traits can be inaccurate. Prior to my study, only 12 genetically-confirmed natural hybrids have been reported (Hillis et al. 1984). These 12 hybrids were initially identified by phenotype and confirmed by electrophoresis, but the level of interspecific admixture within *B. houstonensis* was not determined; consequently, the historic extent of admixture was not

known. Here, the first baseline levels of interspecific genetic admixture in this endangered toad are reported.

When genetic data are used to identify admixture, the probabilistic nature of the analysis results in the estimation of both the type and direction of interspecific hybridization. Determining the level of admixture among species requires choosing some threshold for *Q* where a trade-off between efficiency and accuracy is usually necessary. As defined by Vähä & Primmer (2006), efficiency is the proportion of individuals in a group that were correctly identified, and accuracy is the proportion of an identified group that truly belongs to that category. Using the simulated dataset, the 0.9 threshold was found to be 97.3 % efficient (292 hybrids correctly identified as admixed out of 300 total hybrids) and 98.6 % accurate (292 hybrids correctly identified as admixed out of 296 individuals identified as admixed) at detecting admixed individuals. The 0.85 threshold had higher accuracy but much lower efficiency (99.3 % and 92.7 %). With a 0.9 *Q* value threshold, 117 (117/1112 = 10.5 %) admixed individuals were detected in the experimental dataset, 43 (43/439 = 9.8 %) in phenotypically *Bufo houstonensis* individuals. With an accuracy of 98.6 % there are probably a few more cryptic admixed individuals than were detected here.

An admixture level around 10 % is much higher than reported by Hillis et al. (1984). Less than 1 % of the parental populations in 1981, in part of Bastrop County, Texas, were determined to be hybrids via stepwise discriminant function analysis of morphometric measurements and confirmed to be admixed with allozyme electrophoresis (Hillis et al. 1984). In the present study, the data included samples taken over several

years and from many Texas counties, plus other states and Mexico, making a straightforward comparison with the 1981 data difficult. If individuals from the southern sites in Bastrop County (very near the sites sampled in 1981) from years 2006 and 2007 are considered, the level of admixture may be more easily compared with that from 1981 (Table 9). In 2006, 95 toads were sampled; 2 (2.1 %) were phenotypic hybrids, but 12 (12.6 %) were admixed. In 2007, 139 toads were sampled; 3 (2.2 %) were phenotypic hybrids, but 14 (10.1 %) were admixed. A comparison remains difficult however, because although nearly all *Bufo houstonensis* that were observed were sampled, not all observed *B*. *nebulifer* were sampled. Using only numbers of *B*. *houstonensis* and putative hybrids, a total of 48 and 108 were sampled, 2 and 3 (4.2 % and 2.8 %) were phenotypic hybrids, but 7 and 7 (14.6 % and 6.5 %) were found to be admixed, in 2006 and 2007 respectively. In 1981, out of 1130-2540+ *B*. *houstonensis* and hybrids (numbers of *B*. *houstonensis* are given in ranges by the authors), 12 (<0.5 %-1.1 %) were hybrids (Hillis et al. 1984). The variance and standard deviation of the percentages of phenotypic hybrids from 2006-2007 are both ~0.98, consequently the percentage of hybrids may be as low as 1.82 in 2007. If the variance was similar for the 1981 data, then percentage of hybrids may have been as high as 2.08. Thus, phenotype-based rates of hybridization appear to be stable and low over the past few decades. Nevertheless, phenotype-based rates of hybridization underestimate admixture among species: current phenotype-based estimates of hybridization are around 1 % in all of Bastrop County, whereas genetic-based estimates of admixture are 8.4 % for *B*. *houstonensis* and 7.3 % for *B*. *nebulifer*. Furthermore, 30 (6.7 %) *B*. *houstonensis*-phenotype individuals sampled in Bastrop

County were predefined hybrids regardless of admixture levels; that is, at least 6.7 % were cryptic hybrids. Genetic admixture in *B. houstonensis* may be underestimated by at least 6.7 %. The level of cryptic genetic admixture found here falls within the range reported in other bufonid species: <4 % in *B. fowleri*-*B. nebulifer* (Vogel & Johnson 2008) to 12 %-20 % in *B. americanus*-*B. fowleri*-*B. woodhousii* (Masta et al. 2002).

Laboratory crosses among bufonid taxa indicate that species within the *Bufo americanus* species group (e.g., *B. houstonensis*, *B. microscaphus*, *B. terrestris*, *B. woodhousii*) produce fertile hybrid offspring, with some reduction in fertility depending on the cross (Blair 1959, Kennedy 1961, Blair 1963, Brown 1971b, Blair 1972). *Bufo houstonensis* has been crossed with *B. americanus*, *B. microscaphus*, *B. terrestris*, *B. woodhousii*, and *B. nebulifer* (see Table 10, Thornton 1955, Blair 1959, Kennedy 1961, Blair 1963, Brown 1971b, Blair 1972). It is known that crosses of *B. houstonensis* × *B. woodhousii*, *B. houstonensis* × *B. terrestris*, and reciprocal crosses of the latter can result in fertile F_1 hybrids. Other crossing experiments were terminated before the adult stage was reached, or no backcrosses were attempted to determine the fertility of F_1 hybrids. Crosses of members in the *B. americanus* species group with *B. nebulifer* have also been performed (Thornton 1955, Blair 1959, Kennedy 1961, Blair 1972). Viable offspring can result but usually at lower rates than among crosses within the *B. americanus* species group, and viable offspring are more likely to result when *B. nebulifer* is the paternal parent than when it is the maternal parent (Table 10). No laboratory crosses exist for *B. nebulifer* × *B. houstonensis*, but Thornton (1955) made the cross of *B. nebulifer* × *B. woodhousii*, a close relative of *B. houstonensis*, and the backcross to female *B.*

woodhousii, and found that F_1 hybrids were sterile. Until recently, *B. nebulifer* × *Bufo fowleri* (a member of the *B. americanus* species group) F_1 hybrids were thought to be sterile (Blair 1959), but Vogel & Johnson (2008) provide evidence that these hybrids can be fertile.

In my study, levels of backcrossing could not be determined in NEWHYBRIDS analyses because admixed individuals were all assigned to the F_2 hybrid class. However, other evidence suggests that *Bufo nebulifer* × *B. houstonensis* F_1 hybrids can be fertile and that backcrossing among the three species does occur. F_1 hybrids should have *Q* values near 0.5. Few individuals in this dataset (15 out of 1112) had *Q* values 0.4-0.6, and only 40 had *Q* values 0.3-0.7, indicating that many, if not most, of the admixed individuals are backcrosses or *n*th generation backcrosses. Thirty-two predefined hybrids had *B. houstonensis* phenotypes with *B. nebulifer* or *B. woodhousii* mtDNA haplotypes (Table 4): 6 with *B. nebulifer* haplotypes were assigned to *B. nebulifer* (suggesting that *B. nebulifer* × *B. houstonensis* F_1 hybrids are fertile and have backcrossed to *B. nebulifer*) and 19 with *B. woodhousii* haplotypes were assigned to *B. houstonensis* (suggesting that *B. woodhousii* × *B. houstonensis* F_1 hybrids have backcrossed to *B. houstonensis*). At least two separate hybridization events resulted in the 6 backcrosses to *B. nebulifer*. Individuals MF22053 and MF22121 were adult males sampled in 2002 at a site in Leon County, Texas. The other four individuals were sampled in Bastrop County in 2001 and 2003. MF03650 ($Q_{B.\ nebulifer}$ = 0.920, Table 4) was an adult sampled in February 2001. MF04871 and MF04874 ($Q_{B.\ nebulifer}$ = 0.986 and 0.989 respectively) were juvenile metamorphs, and probably siblings, sampled in June 2001 <1 km from where MF03650

was sampled. MF21332 ($Q_{B.\ nebulifer}$ = 0.991) was a juvenile sampled in June 2003 very near the same site as MF4871 and MF04874. MF03650 may be an ancestor of MF04871, MF04874, and MF21332. If so, then *Q* increasing over time indicates successive backcrosses to *B*. *nebulifer* after the initial hybridization event. Among the predefined hybrids, backcrossing appears to be asymmetric: mean *Q* was higher for the *B*. *houstonensis* cluster (0.659, range = 0.006-0.993 ± 0.104 95 % CI) than for the *B*. *nebulifer* cluster (0.268, range = 0.004-0.991 ± 0.104) or the *B*. *woodhousii* cluster (0.073, range = 0.002-0.608 ± 0.038). When categorized by mtDNA haplotype, predefined hybrids with a *B*. *houstonensis* or *B*. *woodhousii* maternal lineage backcrossed to *B*. *houstonensis* (mean *Q* = 0.83, range = 0.241-0.993 ± 0.072) but hybrids with a *B*. *nebulifer* maternal lineage backcrossed to *B*. *nebulifer* (mean *Q* = 0.882, range = 0.670-0.991 ± 0.078).

Hybrids between *Bufo houstonensis* and *B*. *nebulifer* are reported to be easy to distinguish by morphology and call characteristics (Brown 1971b, Hillis et al. 1984). Hillis et al. (1984) reported that F_1 hybrids have a small amount of yellow pigment of the vocal sacs and are intermediate between the parental species using other morphometric measurements (distance between interocular crests, length of parotoid gland, length of tibiofibula, and distance between parotoid gland and transverse axis of postorbital crest; their Fig. 2). Brown (1971b) states that hybrids between *B*. *houstonensis* and *B*. *nebulifer* are easily identified by round or oval parotoid glands and by their intermediacy in size, color, markings, and cranial crest structure. However, Kennedy (1961) reported that some *B*. *houstonensis* × *B*. *nebulifer* F_1 hybrids resembled *B*. *houstonensis* in general

morphology and others resembled *B*. *nebulifer*. Few putative hybrids in my study had *Q* values near 0.5, suggesting that most putative hybrids were backcrosses and not F_1s (Table 4, compare placement of open squares [F_1s] in Fig. 6 with placement of symbols in Fig. 3). Nine of the 43 admixed *B*. *houstonensis*-phenotype individuals did have *Q* = 0.4-0.6 (32 had *Q* = 0.3-0.7). These results show that not all phenotypically aberrant toads are F_1s and not all F_1s are phenotypically aberrant.

While hybridization/admixture may be cryptic, rates of hybridization seem to be stable over time. However, hybridization rates will quite plausibly rise in the future (see also Allendorf et al. 2001). Both *Bufo houstonensis* (see Chapter 2) and *B*. *woodhousii* (Dixon 2000) are declining in at least part of their ranges in Texas. *Bufo nebulifer*, in contrast, appears to thrive throughout its range, and due to its ability to tolerate habitat alteration, may be expanding its range (Mendelson 2005). Indeed, hybridization with *B*. *nebulifer* may have been a cause in the extirpation of *B*. *woodhousii* in southern Texas (Dixon 2000). Declines of *B*. *woodhousii* have also been observed from the vicinity of Austin, Texas (D. Hillis, personal communication to Sullivan 2005). In areas of sympatry, *B*. *houstonensis* do well competitively when they outnumber other bufonid species, but increasing numbers of *B*. *nebulifer* usually means decreasing numbers of *B*. *houstonensis* (USFWS 2001) and abundance of either *B*. *nebulifer* or *B*. *woodhousii* generally indicates that *B*. *houstonensis* are absent (Yantis 1991).

Historically, the three species were reproductively isolated through three main mechanisms: habitat isolation, offset breeding times, and advertisement call. *Bufo houstonensis* prefer canopied, small, ephemeral water bodies for breeding, while *B*.

nebulifer and *B*. *woodhousii* prefer open areas but use a variety of water bodies to breed (Kennedy 1961, Brown 1971b, Thomas & Potter 1975, Potter et al. 1984, Mendelson 2005, Sullivan 2005). Habitat alteration, especially deforestation, will allow greater opportunities for hybridization events to occur. Breeding seasons of the three species overlap, but the peaks of breeding do not usually coincide (Brown 1971b, Hillis et al. 1984). *Bufo houstonensis* breeds January-June (Kennedy 1961, Brown 1971b, Hillis et al. 1984, Jacobson 1989), *B*. *woodhousii* breeds February-June in central Texas (Thornton 1960, Hillis et al. 1984), and *B*. *nebulifer* breeds March-September in central Texas (Thornton 1960, Hillis et al. 1984). Temperatures are expected to rise in North America (Christensen et al. 2007). Not only will this likely reduce numbers of *B*. *houstonensis* because it is probably a northern-adapted species (Brown 1971b), but warmer temperatures may extend the breeding season of *B*. *nebulifer* into early spring and late winter or affect peak spawning times, causing more overlap with *B*. *houstonensis*. Advertisement call in bufonids is well studied (e.g., Blair 1956a, 1958, McAlister 1961, Porter 1964, Zweifel 1968, Ferguson 1969, Brown 1971b, Thomas & Dessauer 1982, Sullivan et al. 1996, Malmos et al. 2001) and is believed to be a key isolating mechanism, perhaps the most important one (Blair 1962, 1963). *Bufo houstonensis* and *B*. *nebulifer* have trilled calls, but *B*. *houstonensis* calls are a higher frequency (2300 vs. 1250-1800 cycle per sec) and longer duration (3.8-11.2 vs. 1.9-5.0 sec) than *B*. *nebulifer* calls (Blair 1956a). The advertisement call of *B*. *woodhousii* is short (0.8-2.7 sec) and not trilled (Blair 1956a). While advertisement calls appear to be a good premating isolating mechanism, male bufonids are known to clasp (engage in amplexus with) other species

and other males (Blair 1941, Blair 1958, Thornton 1960, Brown 1971a, Waldman et al. 1992), so advertisement call is not a perfect isolating mechanism. In addition, *B*. *nebulifer* females were found to prefer calls of longer duration in conspecific discrimination experiments but did not discriminate between high- and low-frequency calls (Wagner & Sullivan 1995). This may mean that some female *B*. *nebulifer* are attracted to the advertisement call of *B*. *houstonensis*, which are longer but higher in pitch, which may increase the chances of an heterospecific mating. As noted by other authors (Brown 1971b, Hillis et al. 1984), premating isolating mechanisms, except advertisement call, have partially broken down. This trend will continue with expanding habitat alteration and rising temperatures, and these isolating mechanisms may even deteriorate to the point of failure.

Postzygotic isolating mechanisms, like selection against hybrids in the earliest stages of life, generally occur between distantly related taxa in Bufonidae (Blair 1963, 1972, Malone & Fontenot 2008). Malone & Fontenot (2008) found that percentage of fertilized eggs that hatched, number of tadpoles, and percentage of tadpoles that metamorphosed decreased with increasing genetic distance. However, postzygotic isolation could be weak between distantly related species and strong between closely related species (Malone & Fontenot 2008). In my study, selection against hybrids was found in the tadpole life-history stage (Fig. 7), but only among *Bufo houstonensis* and *B*. *woodhousii* individuals, even though *B*. *woodhousii* is more closely related to *B*. *houstonensis* than is *B*. *nebulifer*. This may be explained by the timing of the breeding seasons. Because *B*. *houstonensis* and *B*. *woodhousii* have an extended period in which

both are spawning but *B*. *houstonensis* and *B*. *nebulifer* do not, it is essential for selection against hybrids to occur between the former pair of species and unnecessary between the latter. Another explanation is that most offspring of *B*. *nebulifer* and a *B*. *americanus* species group member usually die early in development, like the gastrula or neurula life-history stages (Table 10), so selection against hybrids at later developmental stages is unnecessary.

With a low frequency of heterospecific hybridization events, gamete wastage is usually minimal and inconsequential. In *Bufo houstonensis*, gamete wastage may be very high due to the following: numbers are low (especially numbers of females, see Chapter 2), numbers of *B*. *nebulifer* are high, admixture is cryptic, premating isolating mechanisms are partially or completely broken down, postzygotic selection against some hybrids is low, hybrids of both crosses can be viable and fertile, and some hybrids backcross to another species. Hybridization in *B*. *houstonensis* has clearly become a larger problem than previous authors asserted, but management strategies like increasing the number of toads through supplementation and habitat conservation (discussed in Chapter 2) will not only directly improve census sizes of remaining populations of *B*. *houstonensis*, they will also alleviate hybridization and its concomitant problems.

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Table 1. Comparison of NEWHYBRIDS analyses. 'Possible' hybrids were individuals that were initially identified as one of the three parental species (and not as a predefined hybrid*) but were assigned to a hybrid class in a preceding NEWHYBRIDS analysis. See Materials and Methods for a more detailed description of the analyses

Taxon	number included in the dataset for each pairwise sub-analysis		
	Bufo houstonensis with *B. nebulifer*	*B. houstonensis* with *B. woodhousii*	*B. nebulifer* with *B. woodhousii*
Analysis A (predefined hybrids excluded)			
B. houstonensis	439	439	—
B. nebulifer	600	—	600
B. woodhousii	—	26	26
predefined hybrids	—	—	—
Analysis B (predefined hybrids included)			
B. houstonensis	439	439	—
B. nebulifer	600	—	600
B. woodhousii	—	26	26
predefined hybrids	47	47	47
Analysis C (predefined and 'possible' hybrids excluded)			
B. houstonensis	423	423	—
B. nebulifer	587	—	587
B. woodhousii	—	25	25
predefined hybrids	—	—	—
'possible' hybrids	—	—	—
Analysis D (predefined and 'possible' hybrids included)			
B. houstonensis	422	422	—
B. nebulifer	587	—	587
B. woodhousii	—	25	25
predefined hybrids	47	47	47
'possible' hybrids	31	31	31

*Predefined hybrid = putative hybrids or individuals with mismatches between phenotype and mtDNA, phenotype and diagnostic alleles, mtDNA and diagnostic alleles, or among diagnostic alleles

Table 2. Hybrid crosses simulated in HYBRIDLAB version 1.0. H = *Bufo houstonensis*, N = *B. nebulifer*, W = *B. woodhousii*, F_1 = cross between parentals, F_2 = cross between F_1s, BX = backcross between a parental and an F_1

	H	N	W	F_1 HN	F_1 HW	F_1 NW
H		F_1 HN	F_1 HW	BX HHN	BX HHW	
N			F_1 NW	BX NHN		BX NNW
W					BX WHW	BX WNW
F_1 HN				F_2 HNHN	F_2 HNHW	F_2 HNNW
F_1 HW					F_2 HWHW	F_2 HWNW
F_1 NW						F_2 NWNW

Table 3. Comparison of assignments by genetic assignment analysis. Numbers of individuals assigned to the following categories are shown: H = assigned to *Bufo houstonensis*, N = assigned to *B. nebulifer*, W = assigned to *B. woodhousii*, and admixed = not assigned to a parental. Percentages are shown below numbers of individuals

STRUCTURE, threshold = 0.9				STRUCTURE, threshold = 0.85				NEWHYBRIDS, threshold = 0.5			
H	N	W	admixed	H	N	W	admixed	H	N	W	admixed
***B. houstonensis* (*n* = 439)**											
396			43	407			32	425			14
90.2 %			9.8 %	92.7 %			7.3 %	96.8 %			3.2 %
***B. nebulifer* (*n* = 600)**											
	548		52	1	564		35	1	590		9
	91.3 %		8.7 %	0.2 %	94 %		5.8 %	0.2 %	98.3 %		1.5 %
***B. woodhousii* (*n* = 26)**											
		25	1			25	1			25	1
		96.2 %	3.8 %			96.2 %	3.8 %			96.2 %	3.8 %
predefined hybrids (*n* = 47)											
20	6		21	22	7		18	25	6		16
42.6 %	12.8 %		44.7 %	46.8 %	14.9 %		38.3 %	53.2 %	12.8 %		34 %
all parentals (*n* = 1065)											
—	—	—	96	—	—	—	68	—	—	—	24
			9 %				6.4 %				2.3 %

Table 4. Characteristics of the 47 predefined hybrids. Individual ID (MF ID), phenotype, mtDNA haplotype, diagnostic alleles, *Q* values and assignments at $Q > 0.9$ resulting from STRUCTURE analysis, and assignments resulting from NEWHYBRIDS analysis D are shown. H = *Bufo houstonensis*, N = *B. nebulifer*, and W = *B. woodhousii*, — = unknown mtDNA haplotype, and * = intermediate advertisement call

MF ID	Phenotype	mtDNA haplotype	Diagnostic alleles	STRUCTURE *Q* values			*Q* threshold = 0.9	NEWHYBRIDS analysis D		
				H	N	W		HN sub-analysis	HW sub-analysis	NW sub-analysis
03619	H	wooA	Nearctic	0.373	0.019	0.608	admixed	H	H	W
03620	H	wooA	Nearctic	0.585	0.007	0.407	admixed	H	admixed	W
03621	H	wooA	Nearctic	0.983	0.006	0.012	H	H	H	W
03622	H	wooA	Nearctic	0.717	0.037	0.246	admixed	H	H	W
03631	H	wooC	Nearctic	0.983	0.01	0.008	H	H	H	W
03650	H	nebB	both	0.076	0.92	0.004	N	N	admixed	N
03651	putative hybrid*	wooC	Nearctic	0.987	0.01	0.003	H	H	H	W
03802	putative hybrid*	wooA	both	0.542	0.455	0.003	admixed	admixed	H	admixed
03803	W	houA	Nearctic	0.729	0.268	0.004	admixed	admixed	H	admixed
03901	W	wooA	Nearctic	0.979	0.018	0.003	H	H	H	admixed
04445	W	wooC	Nearctic	0.99	0.006	0.005	H	H	H	W
04867	H	wooA	Nearctic	0.99	0.005	0.004	H	H	H	W
04869	H	wooC	Nearctic	0.99	0.006	0.004	H	H	H	W
04871	H	nebA	both	0.01	0.986	0.004	N	N	admixed	N
04873	H	nebA	*B. nebulifer*	0.095	0.891	0.014	admixed	admixed	W	N
04874	H	nebA	both	0.008	0.989	0.003	N	N	admixed	N
05718	H	wooA	Nearctic	0.891	0.008	0.101	admixed	H	H	W
05719	H	wooA	Nearctic	0.865	0.038	0.097	admixed	H	H	admixed
05721	H	wooA	Nearctic	0.705	0.012	0.282	admixed	H	admixed	W
05722	H	wooA	Nearctic	0.99	0.007	0.003	H	H	H	W
05723	H	wooA	Nearctic	0.576	0.036	0.388	admixed	H	admixed	W
05724	H	wooA	Nearctic	0.703	0.014	0.283	admixed	H	admixed	W
08911	H	—	both	0.718	0.279	0.003	admixed	H	H	admixed
08997	H	wooA	Nearctic	0.989	0.006	0.005	H	H	H	W
09070	H	wooA	Nearctic	0.993	0.004	0.003	H	H	H	W
09349	H	wooA	Nearctic	0.96	0.014	0.026	H	H	H	W
16653	H	wooA	Nearctic	0.991	0.006	0.003	H	H	H	W
16654	H	wooA	Nearctic	0.992	0.005	0.004	H	H	H	W
16663	putative hybrid*	—	both	0.797	0.175	0.029	admixed	admixed	admixed	W
16716	H	wooA	Nearctic	0.944	0.017	0.04	H	H	H	W
16990	H	nebA	both	0.231	0.728	0.042	admixed	admixed	admixed	N

17099	H	houA	both	0.617	0.374	0.008	admixed	admixed	admixed	W
17100	H	—	both	0.522	0.467	0.012	admixed	admixed	admixed	W
20010	H	woo	Nearctic	0.989	0.006	0.005	H	H	H	W
20057	putative hybrid	houC	both	0.402	0.335	0.264	admixed	admixed	admixed	admixed
20059	putative hybrid	houB	both	0.721	0.276	0.002	admixed	admixed	admixed	admixed
21332	H	nebA	both	0.006	0.991	0.003	N	N	W	N
22053	H	nebF	both	0.018	0.973	0.009	N	N	W	N
22121	H	nebF	both	0.027	0.952	0.021	N	N	W	N
22291	H	woo	Nearctic	0.932	0.007	0.061	H	H	H	W
22322	H	wooA	Nearctic	0.985	0.006	0.009	H	H	H	W
22323	H	wooA	Nearctic	0.988	0.009	0.003	H	H	H	W
22369	putative hybrid	houC	both	0.917	0.076	0.007	H	H	H	W
22419	putative hybrid	nebC	both	0.09	0.67	0.241	admixed	admixed	admixed	admixed
22420	putative hybrid*	houB	both	0.241	0.741	0.019	admixed	admixed	admixed	admixed
22472	H	wooA	Nearctic	0.977	0.021	0.003	H	H	H	W
22498	putative hybrid	nebD	both	0.153	0.724	0.123	admixed	admixed	admixed	admixed

Table 5. Number and percentage of admixed individuals per year by population (*Bufo houstonensis*, see Chapter 2) or area (*B. nebulifer*) in Bastrop County, Texas

	Population/area					
	BAPp	BAS06p	N	S_1	S_2	Total
Bufo houstonensis						
2000			0/2			0/2
2001			4/25 16 %			4/25 16 %
2002			4/55 7.3 %			4/55 7.3 %
2003	0/1	2/7 28.6 %	0/17			2/25 8 %
2004			3/26 11.3 %			3/26 11.3 %
2005	0/5	0/8	5/44 11.4 %			5/57 8.8 %
2006	4/23 17.4 %		0/4	3/35 8.6 %	2/6 33.3 %	9/68 13.2 %
2007	0/10	0/2	1/9 11.1 %	3/31 9.7 %	1/52 1.9 %	5/104 4.7 %
Total	4/39 10.3 %	2/17 11.8 %	17/182 9.3 %	6/66 9.1 %	3/58 5.2 %	32/362 8.8 %
Bufo nebulifer						
2000						
2001			1/13 7.7 %			1/13 7.7 %
2002			0/4			0/4
2003			0/1			0/1
2004			0/18			0/18
2005			0/4			0/4
2006	0/21		2/55 3.6 %	2/29 6.9 %	3/16 18.8 %	7/121 5.8 %
2007			1/24 4.2 %		4/17 23.5 %	5/41 12.2 %
Total	0/21		4/119 3.4 %	2/29 6.9 %	7/33 21.2 %	13/202 6.4 %

Table 6. Comparison of assignments by genetic assignment analysis for the simulated dataset. Numbers of individuals assigned to the following categories are shown: H = assigned to *Bufo houstonensis*, N = assigned to *B. nebulifer*, W = assigned to *B. woodhousii*, and admixed = not assigned to a parental. Percentages are shown below numbers of individuals

STRUCTURE, threshold = 0.9				STRUCTURE, threshold = 0.85				NEWHYBRIDS, threshold = 0.5			
H	N	W	admixed	H	N	W	admixed	H	N	W	admixed
***B. houstonensis* (*n* = 20)**											
18			2	19			1	20			
90 %			10 %	95 %			5 %	100 %			
***B. nebulifer* (*n* = 20)**											
	18		2		19		1		20		
	90 %		10 %		95 %		5 %		100 %		
***B. woodhousii* (*n* = 20)**											
		20				20				20	
		100 %				100 %				100 %	
F_1 hybrids (*n* = 60)											
			60				60	19			41
			100 %				100 %	31.7 %			68.3 %
F_2 hybrids (*n* = 120)											
			120				120	21	1		98
			100 %				100 %	17.5 %	0.8 %		81.7 %
BX hybrids (*n* = 120)											
1	4	3	112	7	7	8	98	28	4	8	80
0.8 %	3.3 %	2.5 %	93.3 %	5.8 %	5.8 %	6.7 %	81.7 %	23.3 %	3.3 %	6.7 %	66.7 %
all parentals (*n* = 60)											
—	—	—	4	—	—	—	2	—	—	—	0
			6.7 %				3.3 %				0 %
all hybrids (*n* = 300)											
1	4	3	292	7	7	8	278	68	5	8	219
0.3 %	1.3 %	1 %	97.3 %	2.3 %	2.3 %	2.7 %	92.7 %	22.7 %	1.7 %	2.7 %	73 %

Table 7. Characteristics of genetic diversity in the three parental *Bufo* species. Sample size (n), number of alleles (A), number of private alleles (A_p), and expected (H_E) and observed (H_O) heterozygosities are provided. Observed heterozygosities followed by a * significantly deviated from HWE before sequential Bonferroni correction; none significantly deviated from HWE after sequential Bonferroni correction

Locus	*B. houstonensis*	*B. nebulifer*	*B. woodhousii*	All
BBR34-2				
n	247	0	21	268
A	24	0	7	27
A_p	20	0	3	23
H_E	0.86092	NA	0.78746	
H_O	0.54251*	NA	0.52381*	
BBR36				
n	382	529	22	933
A	22	16	13	40
A_p	14	9	6	29
H_E	0.91014	0.78419	0.91543	
H_O	0.62565*	0.65028*	0.27273*	
BBR86				
n	0	542	0	542
A	0	10	0	10
A_p	0	10	0	10
H_E	NA	0.51265	NA	
H_O	NA	0.39852*	NA	
BBR281				
n	391	3	15	409
A	9	2	8	12
A_p	2	0	3	5
H_E	0.15292	0.33333	0.73333	
H_O	0.06138*	0.33333	0.26667*	
BC52.03				
n	216	0	25	241
A	9	0	4	10
A_p	6	0	1	7
H_E	0.77473	NA	0.59673	
H_O	0.19907*	NA	0.20000*	
BC52.10				
n	395	341	25	761
A	17	25	8	33
A_p	7	15	0	22
H_E	0.88649	0.71553	0.82041	
H_O	0.55696*	0.35191*	0.64000*	
BC52.12				
n	165	2	19	186
A	10	3	7	14
A_p	5	0	3	8
H_E	0.72794	0.83333	0.77240	
H_O	0.18788*	1.0000	0.42105*	
bco15				
n	396	470	25	891
A	12	1	10	15
A_p	4	1	2	7

H_E	0.86269	NA	0.68898	
H_O	0.71717*	NA	0.32000*	
BM224other				
n	396	547	25	968
A	9	9	5	13
A_p	2	2	2	6
H_E	0.74568	0.73360	0.46286	
H_O	0.59091*	0.54296*	0.36000*	
IHHH				
n	396	0	25	421
A	27	0	11	32
A_p	21	0	5	26
H_E	0.84381	NA	0.85796	
H_O	0.68434*	NA	0.56000*	
IYY				
n	394	0	0	394
A	7	0	0	7
A_p	7	0	0	7
H_E	0.64270	NA	NA	
H_O	0.48731*	NA	NA	
Total				
n	396	548	25	969
A	146	66	73	213
A_p	88	37	25	150
Mean H_E	0.74080	0.65211	0.73728	
Mean H_O	0.46532	0.54617	0.39603	

Table 8. Pairwise F_{ST} values for the three parental *Bufo* species. Significant F_{ST} values are shown in bold

	B. houstonensis (*n* = 439)	*B. nebulifer* (*n* = 596)	*B. woodhousii* (*n* = 26)
B. houstonensis	—		
B. nebulifer	**0.151**	—	
B. woodhousii	**0.168**	**0.248**	—

Table 9. Comparison of hybridization levels in 1981 reported by Hillis et al. (1984) to those found in my study

	1981	2006	2007	2003-2007
All toads				
Putative hybrids	12/1562-3890+ <0.3 %-0.8 %	2/95 2.1 %	3/139 2.2 %	5/259 1.9 %
Admixed	—	12/95 12.6 %	14/139 10.1 %	32/259 12.4 %
Only *Bufo houstonensis* and putative hybrids				
Putative hybrids	12/1130-2540+ <0.5 %-1.1 %	2/48 4.2 %	3/108 2.8 %	5/182 2.7 %
Admixed	—	7/48 14.6 %	7/108 6.5 %	19/182 10.4 %

Table 10. Summary of reported crosses of *Bufo houstonensis* with other species and of *B. nebulifer* with members of the *americanus* species group. unk = reached adult stage but fertility is unknown, — = experiments were terminated before this stage, and ? = no data exist

Parents		Offspring	
Female	Male	Metamorphosis	Adult
Crosses involving *B. houstonensis* (= H)			
H	*B. americanus*	80 % of fertilized eggs[a]	unk[b]
B. americanus	H	68.7 % of tadpoles[c]	—
H	*B. microscaphus*	27 % of fertilized eggs[a]	—
B. microscaphus	H	no crosses exist	
H	*B. nebulifer*	65 % of tadpoles[d]	unk[d]
B. nebulifer	H	no crosses exist	
H	natural hybrid of H and *B. nebulifer*	died at cleavage stage[e]	
H	*B. terrestris*	69 % of fertilized eggs[a]	fertile[b]
B. terrestris	H	90.3 %-98.7 % of tadpoles[c]	fertile[c]
H	*B. woodhousii*	86 % of fertilized eggs[a], developed past this stage[f]	fertile males[f]
B. woodhousii	H	developed past this stage[f]	—
Crosses involving *B. nebulifer* (= N) and members of the *americanus* species group			
N	*B. americanus*	died at gastrula stage[c], 3 % of fertilized eggs[a]	?
B. americanus	N	72 % of fertilized eggs[a]	?
N	*B. houstonensis*	no crosses exist	
B. houstonensis	N	65 % of tadpoles[d]	unk[d]
N	*B. woodhousii*	died at late neurula stage[g]	
B. woodhousii	N	developed past this stage[g]	sterile[g]

[a] Blair 1972
[b] Blair 1963
[c] Blair 1959
[d] Kennedy 1961
[e] Brown 1971b
[f] W.F. Blair personal communication to L.E. Brown in Brown 1971b
[g] Thornton 1955

Fig. 1. a) *Bufo houstonensis* male (MF22273) with thickened cranial crests, elongate parotoid glands, and a dark vocal sac. b) *Bufo nebulifer* male (MF22427) with prominent cranial crests bordering a wide valley between the eyes, triangular parotoid glands, and a yellowish vocal sac. Photo credits: Jacob T. Jackson


a


b

Fig. 2. Two examples of putative hybrid males, both from Bastrop County, Texas. (a) MF22369 with intermediate cranial crests, intermediate parotoid glands, and *B. houstonensis*-like vocal sac coloring. (b) MF22420 with intermediate cranial crests, *B. nebulifer*-like parotoid glands, and intermediate vocal sac coloring. MF22420 also had an intermediate advertisement call. Photo credits: Jacob T. Jackson

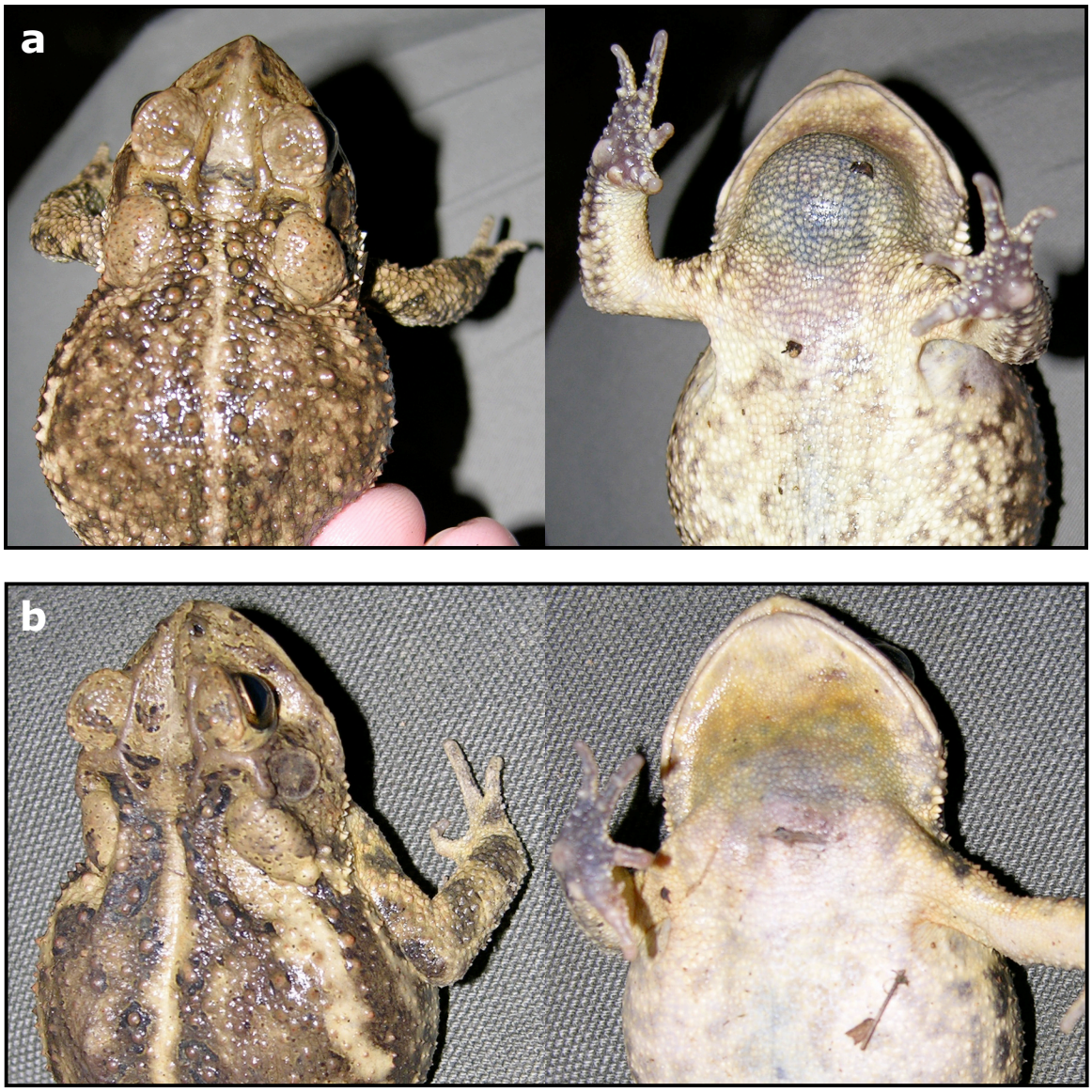

a
b

Fig. 3. Triangle plot of individual proportions of membership in parental species (*Q* values) resulting from analysis in STRUCTURE. Dashed lines at each vertex indicate *Q* value thresholds for that species: black = 0.9 and grey = 0.85. Shape of a symbol indicates the individual's identification prior to any analyses (see Materials and Methods). Individuals that were assigned to the hybrid class by NEWHYBRIDS analysis D are indicated with open red symbols

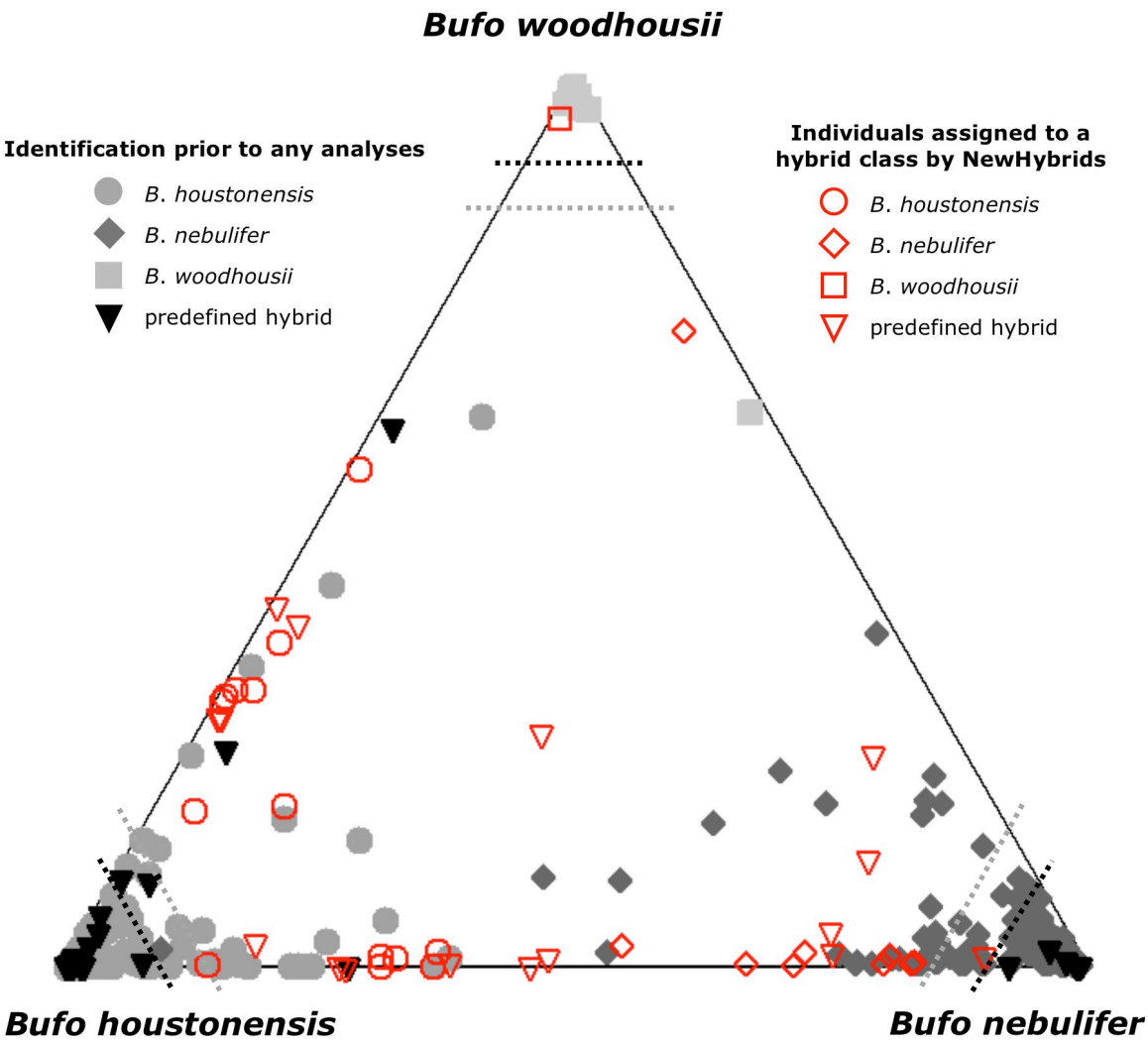
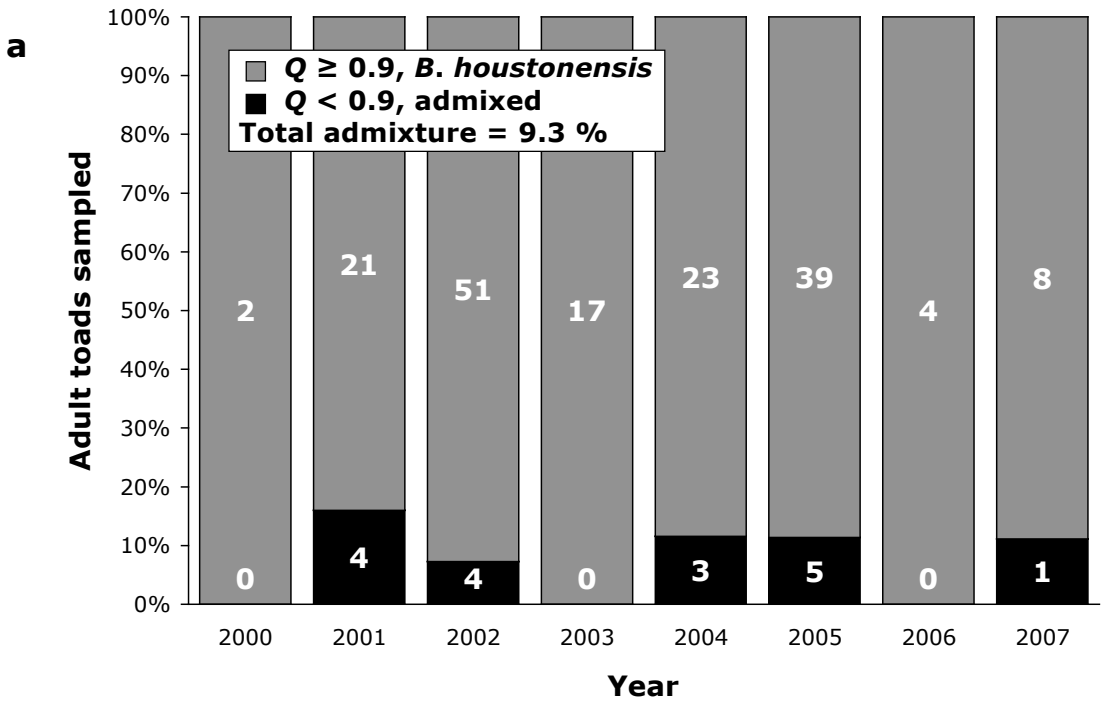

Bufo woodhousii
Identification prior to any analyses
B. houstonensis
B. nebulifer
B. woodhousii
predefined hybrid
Individuals assigned to a hybrid class by NewHybrids
B. houstonensis
B. nebulifer
B. woodhousii
predefined hybrid
Bufo houstonensis
Bufo nebulifer

Fig. 4. Percentage of admixed adult toads per year from population/area N in Bastrop County, Texas (see also Table 5): a) *Bufo houstonensis* phenotype and b) *B*. *nebulifer* phenotype. Admixed individuals had Q values <0.9 and individuals with $Q \geq 0.9$ were assigned to a species


a
Q ≥ 0.9, B. houstonensis
Q < 0.9, admixed
Total admixture = 9.3 %
Adult toads sampled
Year
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
2000
2001
2002
2003
2004
2005
2006
2007
2
21
51
17
23
39
4
8
0
4
4
0
3
5
0
1

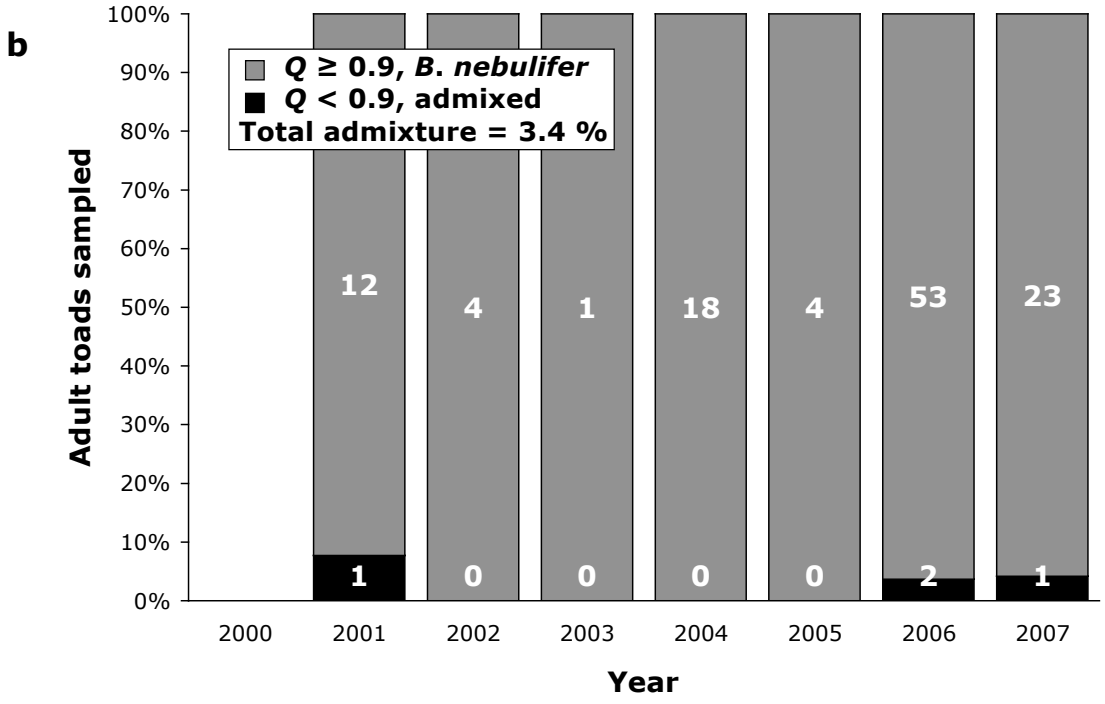
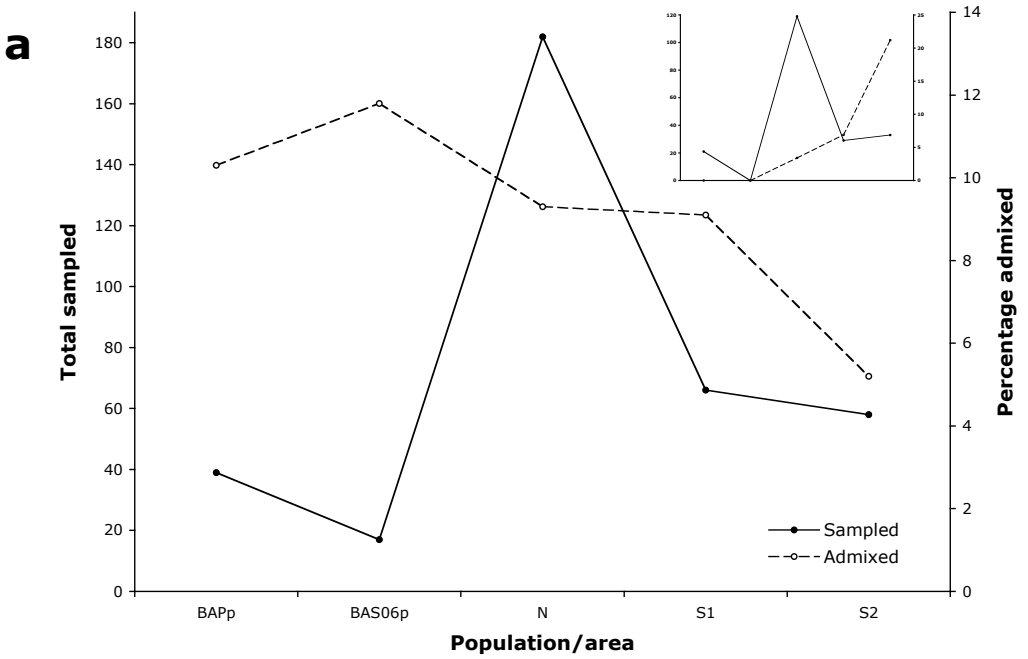

b
Q ≥ 0.9, B. nebulifer
Q < 0.9, admixed
Total admixture = 3.4 %
Adult toads sampled
Year
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
2000
2001
2002
2003
2004
2005
2006
2007
12
4
1
18
4
53
23
1
0
0
0
0
2
1

Fig. 5. a) Total number of individuals sampled (left side y-axis, filled circles and black line) and percentage of individuals that were admixed (right side y-axis, open circles and dashed line) by population for *Bufo houstonensis*. Inset is *B*. *nebulifer*. b) Total number of individuals sampled (left side y-axis, filled circles and black line) and percentage of individuals that were admixed (right side y-axis, open circles and dashed line) by year for *B*. *houstonensis*. Inset is *B*. *nebulifer*


a
Total sampled
Percentage admixed
Sampled
Admixed
BAPp
BAS06p
N
S1
S2
Population/area

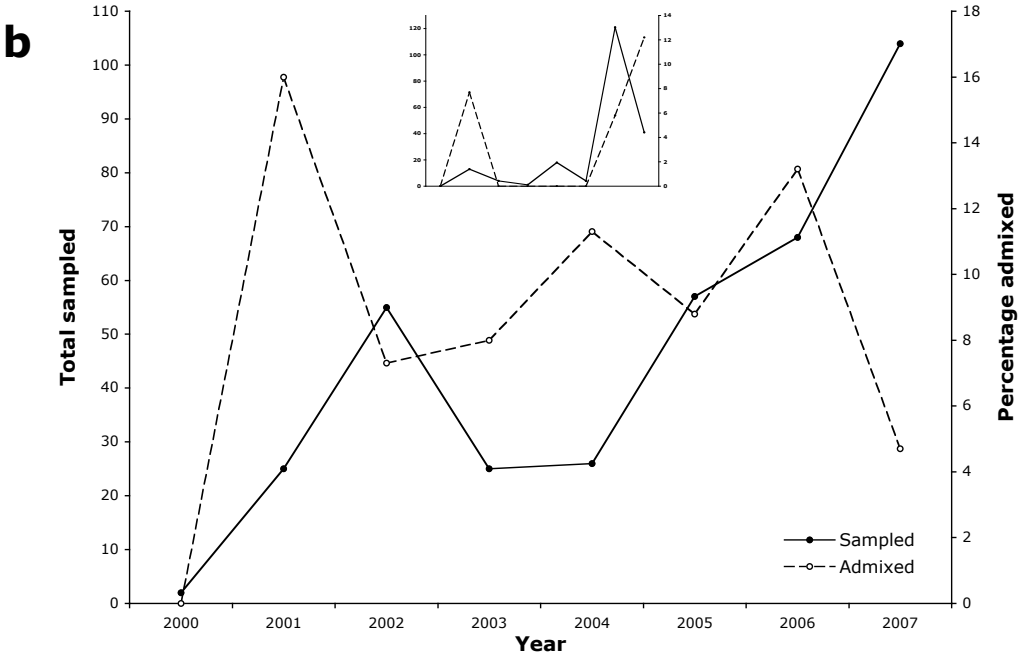

b
Total sampled
Percentage admixed
Sampled
Admixed
2000
2001
2002
2003
2004
2005
2006
2007
Year

Fig. 6. Triangle plot of individual proportions of membership in parental species (*Q* values) resulting from analysis of the simulated dataset in STRUCTURE. Dashed lines at each vertex indicate *Q* value thresholds for that species: black = 0.9 and grey = 0.85. Filled circles are parentals, open squares are F_1s, open diamonds are F_2s, and open circles are BXs. Individuals that were assigned to a hybrid class by NEWHYBRIDS are indicated in red

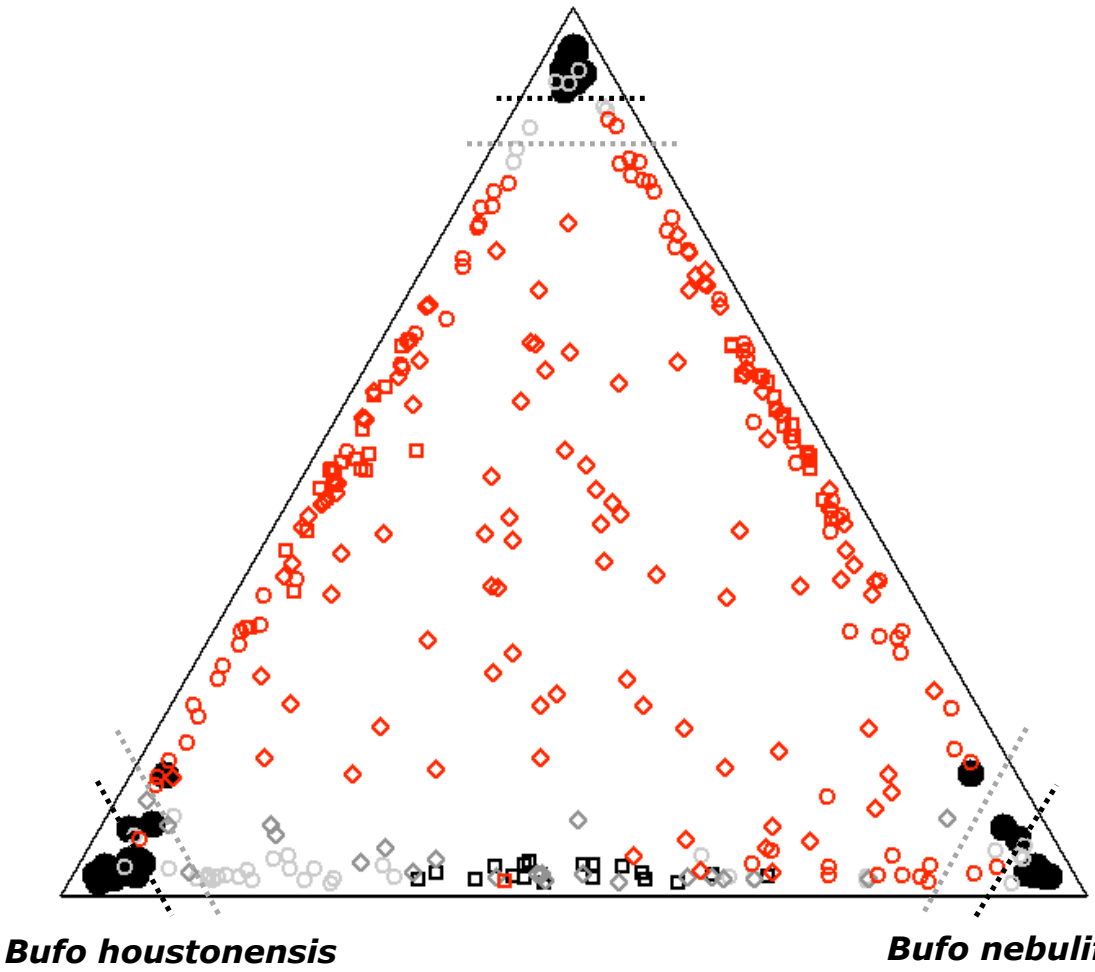
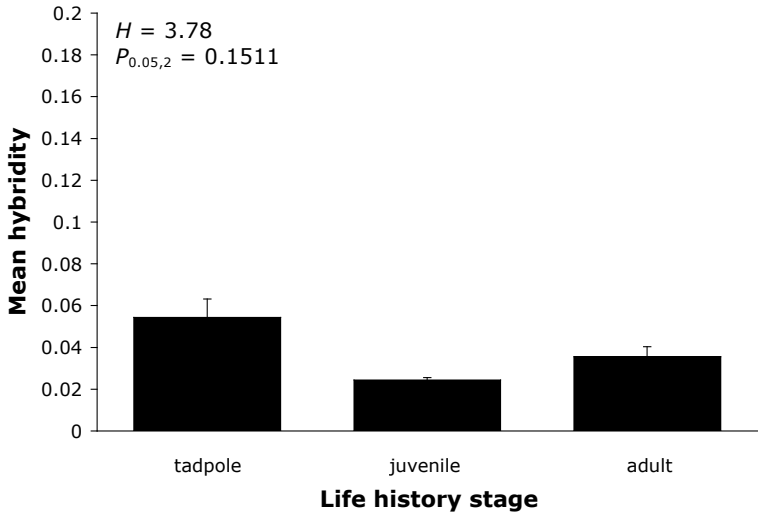
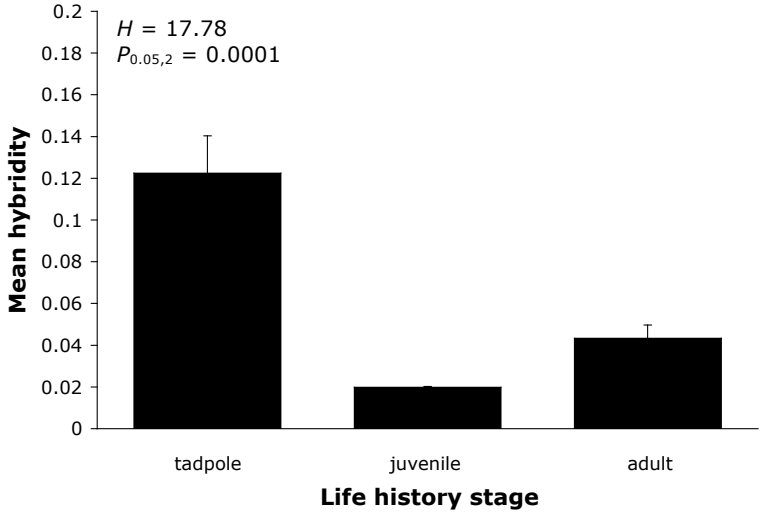

Bufo woodhousii
Bufo houstonensis
Bufo nebulifer

Fig. 7. Mean hybridity (*h*) among three life-history stages: (a) *Bufo houstonensis* and *B. nebulifer*, (b) *B. houstonensis* and *B. woodhousii*. Error bars are variances. Kruskal-Wallis one-way ANOVA results are also presented

a


H = 3.78
P0.05,2 = 0.1511
Mean hybridity
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
tadpole
juvenile
adult
Life history stage

b


H = 17.78
P0.05,2 = 0.0001
Mean hybridity
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
tadpole
juvenile
adult
Life history stage

Chapter 5

CROSS-SPECIES AMPLIFICATION OF BUFONID MICROSATELLITE LOCI IN *BUFO HOUSTONENSIS*, *B. NEBULIFER*, AND *B. WOODHOUSII*

Abstract

Thirty-five published microsatellite loci were screened in several *Bufo* species, chiefly the endangered *Bufo houstonensis* and its two common, sympatric relatives, *B. nebulifer* and *B. woodhousii*. Twelve loci were polymorphic in the three focal species. For some loci, amplification was observed in distantly related species. Natural hybridization occurs within the genus *Bufo* and laboratory crosses often result in viable or fertile offspring. These microsatellite loci may be used to address questions of interspecific admixture as well as baseline intraspecific genetic variation.

Key words: *Bufo houstonensis*, *Bufo nebulifer*, *Bufo woodhousii*, conservation, microsatellite

The ~250 species in the toad genus *Bufo sensu lato* (for recent taxonomic changes within the genus see Frost *et al.* 2006b; Frost *et al.* 2006a; Frost *et al.* 2009) are found nearly world-wide and occupy a broad variety of habitats (Blair 1972b). According to the IUCN Red List of Threatened Species, 31 are Endangered and 10 are Critically Endangered (IUCN 2009). The Houston toad, *Bufo houstonensis*, is endemic to southeast-central

Texas and is listed as endangered at the State and Federal levels (Gottschalk 1970; Potter *et al.* 1984; Campbell 2003). Hybridization among bufonid toads is well known to occur naturally (Blair 1972a). Natural hybrids between *B. houstonensis* and two common and sympatric relatives (*B. nebulifer* and *B. woodhousii*) have been found (Brown 1971; Hillis *et al.* 1984), and laboratory crosses with other bufonid species result in viable or fertile offspring (Blair 1959, 1963, 1972a). Microsatellite loci can be used to measure the genetic variation and structure within a species, which is key in management of endangered taxa such as *B. houstonensis*, but they can also be applied to assess hybridization, or admixture, among species; this is especially important for a group like the genus *Bufo* where admixture among multiple sympatric species may be prevalent.

Thirty-five microsatellite loci from the literature were tested in multiple bufonid species: two loci developed in *Bufo bufo* (Bbuf15, Bbuf49; Brede *et al.* 2001), eight in *Bufo boreas* (BBR16, BBR34-2, BBR36, BBR86, BBR87b, BBR281, BBR292, BBR297; Simandle *et al.* 2006), 16 in *Bufo cognatus* (BC52.03, BC52.04, BC52.10, BC52.11, BC52.12, BC60.20, BC60.35, BC60.37, bco04, bco15, bco40, ICCC, IDDD, IHHH, IKK, IYY; Gonzalez *et al.* 2004; Chan 2007), and nine in *Bufo marinus* (BM121, BM128, BM217, BM218, BM224, BM229, BM239, BM279, BM322; Tikel *et al.* 2000). In addition to the three focal species, *B. houstonensis*, *B. nebulifer*, and *B. woodhousii*, 11 other species in the genus were also screened to evaluate the utility of these markers in New Worlds bufonids (Appendix A).

Toe clip or blood tissue samples were collected from live adult toads (liver or muscle was taken from dead animals). Blood samples were stored at –80 °C in a blood

storage buffer modified from Longmire et al. (1988): 100 mM TRIS, 100 mM EDTA disodium dihydrate, 1 % w/v sodium dodecyl sulfate, pH = 8.0. Toe clips, liver, and muscle were stored in 96 % ethanol at –80 °C. Tissues were deposited in the Michael R. J. Forstner Frozen Tissue catalog at Texas State University—San Marcos. Vouchered specimens were deposited at the Texas Cooperative Wildlife Collection (TCWC84123, TCWC84579) and Coleccion de Herpetología, Escuela de Biología, Universidad de Costa Rica (UCR15632, UCR15633, UCR15722).

DNA was isolated from tissue (1-2 mm^3 toe clip, liver, or muscle; 10-50 μl blood in storage buffer) using a Wizard® SV 96 Genomic DNA Purification System (Promega) on a Biomek® 3000 Laboratory Automation Workstation (Beckman Coulter), or using a DNeasy® Tissue Kit (QIAGEN Inc.), following manufacturer's instructions for both, or using a standard phenol-chloroform method (Sambrook *et al.* 1989). DNA extractions were assessed by agarose gel electrophoresis and were visualized following ethidium bromide staining under UV light.

Amplifications of microsatellite loci were performed using WellRED fluorescently labeled forward primers in 10 μl reactions with 4 mmol $MgCl_2$, 0.1 mM dNTPs, 0.01 μM each primer, 2.5 units *Taq* polymerase, and pH = 8.5. A range of $MgCl_2$ concentrations was not tested because annealing temperature has been shown to be more important in cross-species amplification (Morin *et al.* 1998). PCR was performed with an initial denaturing period of 95 °C for 5 min then 35 cycles, each consisting of denaturing at 95 °C for 30 sec, annealing for 1 min, and extension at 72 °C for 1 min, and a final extension period of 72 °C for 5 min (except BBR34-2 for which no initial 5 min

denaturing period was used). All loci were tested at 55 °C annealing temperature and all except Bbuf15 and IKK were tested at 50 °C. Seven loci were also tested at 45 °C (BC52.11, BC60.20, BC60.35, BM279, BM322, IKK, IYY), 14 at 60 °C (Bbuf15, Bbuf49, BC52.04, BC52.10, BC52.11, BC52.12, BC60.35, bco15, bco40, BM279, BM322, IHHH, IKK, IYY), and two at 65 °C (Bbuf15, IHHH). Additionally, thermal profiles from the original references were attempted for BC60.20, bco40, and BM121. Amplifiable loci (see below) performed best at 55 °C except IHHH, which performed best at 60 °C, and BC52.10, which in *B*. *nebulifer* performed best at 50 °C. Amplification products were electrophoresed on a CEQ™ 8800 Genetic Analysis System (Beckman Coulter) following manufacturer's instructions. Allele sizes were determined with CEQ™ 8800 FRAGMENT ANALYSIS software (Beckman Coulter) by eye. At least two PCR attempts were made, for each individual per locus, before identifying the locus as not amplifiable. Positive controls were used for loci developed in *B*. *cognatus* and *B*. *marinus*. All 35 loci were screened in *B*. *cognatus* ($n = 1$), *B*, *houstonensis* ($n \geq 2$), *B*. *marinus* ($n \geq 6$, except BBR86 where $n = 1$), *B*. *nebulifer* ($n \geq 2$), and *B*. *woodhousii* ($n = 1$). Following initial screening, 11 loci (BBR34-2, BBR36, BBR86, BBR281, BC52.03, BC52.10, BC52.12, bco15, BM224, IHHH, IYY) were chosen to test more thoroughly in *B*. *americanus*, *B*. *fowleri*, *B*. *houstonensis*, *B*. *nebulifer*, and *B*. *woodhousii*, and to test in other species (*B*. *baxteri*, *B*. *debilis*, *B*. *punctatus*, *B*. *speciosus*, *B*. *coccifer*, *B*. *melanochlorus*, and *B*. *valliceps*).

BM224 was revealed to be an interrupted locus, such that one motif, $(TG)_n$, was separated from the other, $(AG)_n$, by 35 bp of sequence. After sequencing multiple

individuals per allele per species, electromorph size homoplasy was detected (Adams *et al.* 2004). Accordingly, an internal reverse primer (5'-GCTCGCTCAGAGGCTCACTTTGT-3') was developed for use with the forward primer BM224F (Tikel *et al.* 2000); this is the 'BM224RDJM locus'. The length of the second half of BM224 (= locus 'BM224other') was determined by subtracting the allele length of BM224RDJM from the allele length of BM224.

Twelve published loci, plus BM224RDJM and BM224other, were polymorphic in the three focal species. Twenty-four loci were either not amplifiable, monomorphic, or otherwise unsuitable (see below); results from these loci are presented in Table 1. For some loci, despite observable polymorphism, a stringent evaluation conducted via sequence verification revealed that the polymorphisms were the result of indels rather than changes in repeat number. BBR16 was orthologous in *B*. *houstonensis* (monomorphic) and *B*. *marinus* (polymorphic). Bbuf15 was orthologous in four species, but in three, polymorphism resulted from an indel. BC60.20 was orthologous in *B*. *marinus* (few repeats) and *B*. *woodhousii* (monomorphic). BC60.37 was orthologous in *B*. *houstonensis* (polymorphism resulted from an indel) and *B*. *nebulifer* (polymorphic) and is a possible microsatellite in *B*. *woodhousii*; this locus, though polymorphic in *B*. *nebulifer*, was excluded from further analysis because of the indel polymorphism in *B*. *houstonensis*. ICCC was orthologous in *B*. *houstonensis* (monomorphic) and *B*. *woodhousii*. For several loci, multiple and/or nonhomologous bands were amplified or no amplification resulted: BBR297, Bbuf49, BC52.04, BC52.11, BC60.35, bco40, BM121, BM128, BM217, BM229, BM239, BM279, BM322, and IKK. Possible microsatellites

were detected in BBR87b, BBR292, bco04, BM218, and IDDD. Additionally, Bbuf15, BC60.20, and BM128 amplified in *B. fowleri*, and Bbuf15 (HM021038) and ICCC (HM021081) were determined to be orthologous in *B. americanus*.

Repeat motifs and GenBank Accession Numbers for the 11 polymorphic loci (excluding BC60.37), plus BM224RDJM and BM224other, are presented in Table 2. No amplification products were observed for *B. nebulifer* for BBR34-2, BBR281, BC52.12, bco15, IHHH, and IYY. However, a nonhomologous 115 bp fragment was amplified in *B. nebulifer* for bco15 (HM021065); this allele may be used as a species-specific allele in hybridization and introgression studies involving *B. nebulifer*. BBR86 amplified only in *B. nebulifer*. *Bufo woodhousii* did not amplify for IYY. Most motifs were the same as in the source species. A compound motif was observed in BBR36 for *B. americanus*, *B. fowleri*, and *B. houstonensis* and in BC52.03 for *B. americanus*, *B. fowleri*, and *B. woodhousii*. For IYY, a orthologous fragment was detected in *B. americanus* and *B. houstonensis* but with a different repeat motif ($[GTAT]_n$) from the source species ($[GT]_n$); IYY was also probably a tetranucleotide in *B. fowleri*, but this was not sequence verified. As already discussed, electromorph size homoplasy was detected for BM224 in *B. houstonensis* and *B. nebulifer* and may be present in other species. Amplification and homology of BM224 was observed in a *Gastrophryne* sp. (HM021066) and a *Scaphiopus* sp. (HM021068).

To characterize these 13 loci, five *B. americanus* (junction of Dale Road and Highway 165, Taney Co., Missouri; 01 May 2003), five *B. fowleri* (near junction of 218 and 203, Stafford Co., Virginia; 27 Jul 2004), 32 *B. houstonensis* (Griffith League Ranch

pond 2, Bastrop Co., Texas; Feb-Mar 2005), 27 *B*. *nebulifer* (Bastrop State Park pond 19, Bastrop Co., Texas; 26 Apr 2006), and 21 *B*. *woodhousii* (near junction of FM-933 and FM-934, Hill Co., Texas; 2001, 2004, 2006, and 2007) were used (Table 3). Number of alleles per locus (*A*), observed (H_O) and expected (H_E) heterozygosities, and linkage disequilibrium were determined in ARLEQUIN 3.11 (Goudet 2001). All loci were polymorphic in at least one species with between 1 and 12 alleles per species. Six loci (BM224 and BM224other in *B*. *americanus*, BBR36 and bco15 in *B*. *fowleri*, BC52.10 and BM224other in *B*. *nebulifer*) deviated significantly from Hardy-Weinberg equilibrium after sequential Bonferroni correction (adjusted P value = 0.00385; Rice 1989). Some linkage disequilibrium was found (data not shown): three pairs of loci (out of 78 pair-wise combinations) in *B*. *americanus*, 12 in *B*. *fowleri*, 16 in *B*. *houstonensis*, three in *B*. *nebulifer*, and 13 in *B*. *woodhousii*. Excluding BM224 and BM224RDJM, 40 out of 43 (93 %) heterologous species-locus amplifications were polymorphic.

For these 13 loci, multiple other species were screened (Table 4). Excluding BM224 and BM224RDJM, out of 86 heterologous species-locus combinations, 27 (31.4 %) amplifications were detected, of which 15 (17.4 %) were polymorphic. Homology was confirmed in seven species-locus combinations. For IYY, *B*. *punctatus* has the same repeat motif as *B*. *houstonensis* whereas *B*. *speciosus* has the same motif as *B*. *cognatus*. The same 115 bp fragment was detected in *B*. *valliceps* (HM021060) as in *B*. *nebulifer* for bco15.

This study provides researchers with several polymorphic microsatellite loci from which cross-species amplification may be obtained for many bufonid species. These loci

may be especially useful in North American species, and specifically in genetic assessments of the endangered taxa *B. baxteri*, *B. californicus*, *B. canorus*, *B. houstonensis*, and *B. nelsoni*.

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Table 1 Twenty-four loci screened in the three focal species (*Bufo houstonensis*, *B. nebulifer*, and *B. woodhousii*), *B. cognatus*, and *B. marinus*. Amplification was attempted in at least one individual per species (NT = not tested, — = multiple and/or nonhomologous bands were amplified or no amplification). Results of mono- or polymorphism are provided where tested. GenBank Accession Nos. are in parentheses.

Locus	*B. cognatus*	*B. houstonensis*[a]	*B. marinus*[b]	*B. nebulifer*[c]	*B. woodhousii*[d]
BBR16	—	orthologous but monomorphic (HM021025)	orthologous and polymorphic (HM021024)	—	—
BBR87b	—	—	—	—	possible microsat
BBR292	—	—	possible microsat	—	—
BBR297	—	—	—	—	—
Bbuf15	orthologous (HM021037)	orthologous but polymorphism was result of indel (HM021039)	—	orthologous but polymorphism was result of indel (HM021035)	orthologous but polymorphism was result of indel (HM021032)
Bbuf49	NT	—	NT	—	NT
BC52.04	source species	—	—	—	—
BC52.11	source species	—	—	—	—
BC60.20	source species	—	orthologous but few repeats (HM021054)	—	orthologous but monomorphic (HM021053)
BC60.35	source species	—	—	—	—
BC60.37	source species	orthologous but polymorphism was result of indel (HM021055, HM021056)	—	orthologous and polymorphic (HM021057)	possible microsat
bco04	source species	possible microsat	—	—	possible microsat
bco40	source species	—	—	—	—
BM121	—	—	source species	—	—
BM128	—	—	source species	—	—
BM217	—	—	source species	—	—
BM218	possible microsat	—	source species	—	—
BM229	—	—	source species	—	—
BM239	—	—	source species	—	—
BM279	—	—	source species	—	—
BM322	—	—	source species	—	—
ICCC	source species	orthologous but monomorphic (HM021082)	—	—	orthologous (HM021080)
IDDD	source species	—	possible microsat	—	possible microsat
IKK	source species	—	—	—	—

[a]Polymorphism was tested with at least 12 individuals.
[b]Polymorphism was tested with at least six individuals.
[c]Polymorphism was tested with at least 59 individuals.
[d]Polymorphism was tested with at least 19 individuals.

Table 2 Repeat motifs and GenBank Accession Nos. for the 13 polymorphic microsatellite loci in the three focal species (*Bufo houstonensis*, *B. nebulifer*, and *B. woodhousii*), *B. americanus*, and *B. fowleri*.

Locus	Published motif	Motif	GenBank Accession Nos.
BBR34-2[a]	$(TTA)_n$	$(TTA)_n$	HM021026[b]
BBR36	$(TAGA)_n$	$(TAGA)_n$	HM021028[c]
		$(TAGA)_n(CAGA)_n(TAGA)_n$	HM021027, HM021029, HM021030[d]
BBR86[e]	$(ATT)_n$	$(ATT)_n$	HM021031
BBR281[a]	$(AAT)_n$	$(AAT)_n$	HM021032–HM021034[f]
BC52.03[a]	$(TAGA)_nTGGG(TAGA)_n$	$(TAGA)_n$	HM021043[g]
		$(TAGA)_n(CAGA)_n$	HM021040–HM021042
BC52.10	$(GATA)_nGAT(GATA)_n$	$(GATA)_n$	HM021044–HM021047
BC52.12[a]	$(GATA)_n(A)_n$	$(GATA)_n$	HM021048, HM021050–HM021052
bco15[a]	$(TCTA)_n$	$(TCTA)_n$	HM021058, HM021059, HM021062, HM021061
BM224	$(TG)_5TA(TG)_5TA(TG)_2...(AG)_n$	$(TG)_n...(AG)_n$	HM021067, HM021069–HM021071
BM224RDJM	n/a	$(TG)_n$	HM021076–HM021079
BM224other	n/a	$(AG)_n$	HM021072–HM021075
IHHH[a]	$(AC)_n$	$(AC)_n$	HM021085–HM021088
IYY[i]	$(GT)_n$	$(GTAT)_n$	HM021091–HM021092[j]

[a]Did not amplify in *B. nebulifer*.
[b]*B. fowleri*.
[c]*B. nebulifer*.
[d]*B. americanus*, *B. fowleri*, and *B. houstonensis*.
[e]Amplified in only *B. nebulifer*.
[f]*B. americanus*, *B. houstonensis*, and *B. woodhousii*.
[g]*B. houstonensis*.
[h]*B. americanus*, *B. fowleri*, *B. houstonensis*, and *B. woodhousii*.
[i]Did not amplify in *B. nebulifer* or *B. woodhousii*.
[j]*B. americanus* and *B. houstonensis*.

Table 3 Summary statistics of the 13 polymorphic microsatellite loci in the three focal species (*Bufo houstonensis*, *B*. *nebulifer*, and *B*. *woodhousii*), *B*. *americanus*, and *B*. *fowleri* (number tested per species is in parentheses). Number of individuals that amplified (*n*), number of alleles (*A*), and expected (H_E) and observed (H_O) heterozygosities are provided. Observed heterozygosities followed by a * significantly deviated from HWE before sequential Bonferroni correction, and those in bold significantly deviated after correction.

Locus	*B. americanus* (5)					*B. fowleri* (5)					*B. houstonensis* (32)				
	Size range	*n*	*A*	H_E	H_O	Size range	*n*	*A*	H_E	H_O	Size range	*n*	*A*	H_E	H_O
BBR34-2	163-190	4	5	0.875	0.500	166-214	4	5	0.857	1.000	160-205	12	9	0.779	0.417*
BBR36	169-305	5	6	0.889	1.000	221-261	5	5	0.756	**0.400***	169-341	28	12	0.849	0.429*
BBR86	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BBR281	145-157	5	4	0.533	0.600	148-187	4	4	0.750	0.500	145-157	31	4	0.095	0.065*
BC52.03	383-435	5	4	0.644	0.600	387-391	4	2	0.250	0.250	391-411	23	6	0.734	0.174*
BC52.10	163-227	5	5	0.844	0.800	179-199	2	4	1.000	1.000	171-207	32	9	0.831	0.250*
BC52.12	268	1	1	n/a	n/a	272	1	1	n/a	n/a	236-268	20	3	0.655	0.100*
bco15	250-270	5	4	0.733	0.800	246-266	5	4	0.733	**1.000***	234-266	32	8	0.762	0.625*
BM224	145-147	5	2	0.533	**0.000***	143-153	5	4	0.644	0.400	131-161	32	11	0.779	0.563*
BM224RDJM	79	5	1	n/a	n/a	79	5	1	n/a	n/a	67-79	32	4	0.177	0.188*
BM224other	66-68	5	2	0.533	**0.000***	64-74	5	4	0.644	0.400	62-82	32	9	0.732	0.563*
IHHH	177-283	5	6	0.889	0.800	183-219	5	8	0.956	1.000	179-235	32	12	0.794	0.531*
IYY	313-325	5	4	0.711	0.800	321	1	1	n/a	n/a	317-329	32	3	0.624	0.406*
Average *A*			3.7					3.6					7.6		

Table 3 continued

Locus	*B. nebulifer* (27)					*B. woodhousii* (21)					Average *A*	Published *A*	Published size range
	Size range	*n*	*A*	H_E	H_O	Size range	*n*	*A*	H_E	H_O			
BBR34-2	—	—	—	—	—	190-229	17	6	0.749	0.471*	6.3	6	184-205
BBR36	169-217	27	9	0.826	0.741	173-345	17	9	0.882	0.176*	8.4	11	177-221
BBR86	336-345	27	3	0.514	0.444	—	—	—	—	—	3.0	6	149-165
BBR281	—	—	—	—	—	145-175	12	7	0.678	0.333*	4.8	7	139-156
BC52.03	—	—	—	—	—	387-427	21	5	0.624	0.238*	4.3	22	350-528
BC52.10	195-271	15	5	0.618	**0.400***	183-207	21	7	0.810	0.762*	6.0	12	157-209
BC52.12	—	—	—	—	—	244-300	19	7	0.784	0.421*	3.0	31	257-408
bco15	—	—	—	—	—	242-446	21	7	0.512	0.333*	5.8	12	298-370
BM224	141-153	27	7	0.778	0.667	145-157	21	5	0.440	0.429	5.8	5	142-154
BM224RDJM	79-81	27	2	0.453	0.519	77-79	21	2	0.048	0.048	2.0	n/a	n/a
BM224other	60-72	27	7	0.788	**0.593***	66-78	21	4	0.438	0.429	5.2	n/a	n/a
IHHH	—	—	—	—	—	171-217	21	10	0.836	0.619	9.0	33	162
IYY	—	—	—	—	—	—	—	—	—	—	2.7	24	187
Average *A*			5.5					6.3					

— = multiple and/or nonhomologous bands were amplified or no amplification.

Table 4 Results of the 13 polymorphic loci for other *Bufo* species (NT = not tested; — = multiple and/or nonhomologous bands were amplified or no amplification). Number tested (*n*), alleles recovered, and GenBank Accession Nos. are provided.

Locus	North American species (*n* = 1 for each species)				
	B. baxteri	*B. cognatus*	*B. debilis*	*B. punctatus*	*B. speciosus*
BBR34-2	—	187, 193	205	259, 319	—
BBR36	—	—	—	—	—
BBR86	—	369	—	—	342, 369
BBR281	169	148, 163	—	—	163, 169
BC52.03	NT	403, 431	—	—	395, 411
BC52.10	—	175	—	—	—
BC52.12	—	308	—	—	292, 320 (HM021049)
bco15	242, 322 (HM021064)	438, 454	—	—	—
BM224	151	145, 157	149, 153	147, 151	145, 153
BM224RDJM	79	79	79	65, 79	79
BM224other	72	66, 78	70, 74	72, 82	66, 74
IHHH	213	173, 175	—	179 (HM021084)	179 (HM021083)
IYY	—	199	—	329[a] (HM021090)	193 (HM021089)

Table 4 continued

Locus	Central and South American species			
	B. coccifer (*n* = 1)	*B. marinus* (*n* = 23)	*B. melanochlorus* (*n* = 1)	*B. valliceps* (*n* = 1)
BBR34-2	—	184, 190, 193, 196[b]	—	NT
BBR36	—	—	169	—
BBR86	—	—[c]	—	—
BBR281	NT	—	NT	NT
BC52.03	NT	—	NT	NT
BC52.10	—	—	—	247
BC52.12	—	—	—	NT
bco15	NT	382, 386, 390, 398, 402[b] (HM021063)	NT	—
BM224	145, 147	145, 147, 149, 151[d]	157	151
BM224RDJM	79	NT	79	79
BM224other	66, 68	NT	78	72
IHHH	NT	NT	NT	NT
IYY	—	—	—	NT

[a]Repeat motif was $(GTAT)_n$ not $(GT)_n$.
[b]Tested in six individuals.
[c]Tested in one individual.
[d]Tested in 22 individuals.

Chapter 6

SUMMARY

Chapter 2: Microsatellite and mtDNA analyses reveal high genetic diversity and multiple populations of the Houston toad (*Bufo houstonensis*)

1. Using DNAs from tissues sampled across the range of *Bufo houstonensis*, an endangered Texas endemic, and traditional population genetics analyses, I determined how many populations exist, the genetic diversity within and among populations, and the patterns of gene flow at landscape- and fine-scale levels. I also investigated the phylogenetic relationship of *B*. *houstonensis* within the *B*. *americanus* species group.
2. From 439 samples, nine groups, or populations, were found across the range. Five occurred in Bastrop County, where the largest numbers of *B*. *houstonensis* are found; these showed lower levels of differentiation than populations separated by larger geographic distances. The population in Austin County was the most divergent. Gene flow was generally low, but higher at distances <4 kilometers. Overall, genetic variation was high among and within populations.
3. *Bufo houstonensis* was most closely related to *B*. *americanus* and appears to have diverged far earlier than the post-Pleistocene. Fourteen mitochondrial haplotypes were recovered in *B*. *houstonensis*.

4. Results indicated that toads from the 2007 Colorado County sampling site are probably descendants of individuals that were translocated from Bastrop County to the Attwater Prairie Chicken National Wildlife Refuge, Colorado County, during the 1980s.
5. Recent auditory surveys indicate that population sizes are low and decreasing: as of 2008, *B. houstonensis* was found in only five of the historic twelve counties. Accordingly, annual monitoring of all known populations and increasing the number of toads (e.g., through supplementation programs like headstarting) are proposed for immediate implementation. More general, but crucial, recommendations include preservation of all three habitat types (breeding/nursery, occupied, and dispersal), special attention towards the Austin County population, and involvement of the general public in conservation of *B. houstonensis*.

Chapter 3: Genetic variation and population structure in the coastal plain toad (*Bufo nebulifer*)

1. Using 596 DNAs sampled across much of the range of *Bufo nebulifer*, a common and sympatric relative of *B. houstonensis*, and traditional population genetics analyses, I determined how many populations exist, the genetic diversity within and among populations, and the patterns of migration or dispersal at landscape- and fine-scale levels.
2. Nine populations were recovered. Their relationships may be explained by a long residence in much of its present-day distribution (at least tens of

thousands of years), with a history of range contraction during glaciation and re-expansion following the retreat of the glaciers during the Pleistocene.

3. *Bufo houstonensis* and *B*. *nebulifer* had comparable levels of genetic diversity, but *B*. *nebulifer* seems to migrate less frequently or over less distance than its endangered congener.

Chapter 4: Characterizing natural levels of interspecific admixture in an endangered toad

1. Interspecific genetic admixture in the endangered *B*. *houstonensis* with *B*. *nebulifer* and with *Bufo woodhousii* was detected using 439 *B*. *houstonensis* samples, 600 *B*. *nebulifer*, and 26 *B*. *woodhousii*. The latter two species are common and are sympatric with *B*. *houstonensis* throughout its range.
2. Phenotype-based assessments of admixture appear to be temporally stable, but they underestimate true levels of genetic admixture. Admixture was found in multiple populations of *B*. *houstonensis*.
3. *Bufo nebulifer* × *B*. *houstonensis* F_1 hybrids can be fertile and backcross to *B*. *nebulifer*; *B*. *nebulifer* × *B*. *houstonensis* matings may result in fertile offspring more frequently than generally assumed or previously reported.
4. Admixed individuals that have *B*. *houstonensis* or *B*. *woodhousii* maternal lineages can backcross to *B*. *houstonensis*.
5. Phenotypically aberrant individuals were not always F_1 hybrids, and F_1 hybrids were not always phenotypically aberrant.
6. Selection against hybrids at the tadpole stage did not occur among *B*.

houstonensis and *B*. *nebulifer* individuals. With continued habitat alteration and rising temperatures, both habitat isolation and offset breeding season have already partially broken down and may deteriorate further; consequently, opportunities for hybridization events will increase. All these factors may lead to higher levels of gamete wastage in *B*. *houstonensis*, an already critically endangered species.

Chapter 5: Cross-species amplification of bufonid microsatellite loci in *Bufo houstonensis*, *B*. *nebulifer*, and *B*. *woodhousii*

1. Thirty-five published microsatellite loci were screened in several *Bufo* species, chiefly the endangered *B*. *houstonensis* and its common, sympatric relatives, *B*. *nebulifer* and *B*. *woodhousii*.
2. Twelve loci were polymorphic in the three focal species.
3. For some loci, amplification was observed in distantly related species.
4. Natural hybridization occurs within the genus *Bufo*, and laboratory crosses often result in viable or fertile offspring. These microsatellite loci may be used to address questions of interspecific admixture as well as baseline intraspecific genetic variation.
5. These loci may be especially useful in North American species, and specifically in genetic assessments of the endangered taxa *Bufo baxteri*, *B*. *californicus*, *B*. *canorus*, and *B*. *nelsoni*.

Appendix A

MF no. (Michael R. J. Forstner Frozen Tissue catalog identification number), mtDNA haplotype, sex, date sampled, coordinates (WGS84 datum), country, state, county, site code, locality description, and GenBank Acccession No(s). for all individuals used in my research. *Bufo houstonensis* are listed first, then *B*. *nebulifer*, *B*. *woodhousii*, putative hybrids, and predefined hybrids. Other species follow the predefined hybrids and are listed in alphabetical order. The terminal letter in a site code represents the type of site: p = pond, s = site, and t = trap. Locality abbreviations are BBHQ = Bluebonnet Headquarters, BSP = Bastrop State Park, and GLR = Griffith League Ranch.

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
Bufo houstonensis												
3618	houA		02/13/2001	30.30689	-97.16639	USA	Texas	Bastrop	BAN27s	Kuhl site, Road side	2, 4	HM021096
3624		female	06/03/2000	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3625		female	06/03/2000	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3626		female	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3627		female	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3628		male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3629		male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3630		male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3632		male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3633		male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3634		male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3635		male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3636		male	02/23/2001	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
3637		male	02/23/2001	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
3638		female	02/23/2001	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	2, 4	
3639	houC	male	02/23/2001	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	2, 4	HM021097
3647	houA		02/13/2001	30.30689	-97.16639	USA	Texas	Bastrop	BAN27s	Kuhl site, Road side	2, 4	
3648	houB		02/13/2001	30.30689	-97.16639	USA	Texas	Bastrop	BAN27s	Kuhl site, Road side	2, 4	
3655		female	03/29/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
4405		male	02/14/2001	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5A	2, 4	
4529	houA		02/13/2001	30.30689	-97.16639	USA	Texas	Bastrop	BAN27s	Kuhl site, Road side	2, 4	
4792		female	05/03/2001	30.20008	-97.22266	USA	Texas	Bastrop	BAN18t	GLR 10-1	2, 4	
4820		male	03/30/2001	30.21036	-97.23828	USA	Texas	Bastrop	BAN15t	GLR 4	2, 4	
4868	houG	juvenile	05/26/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	HM021098
4875	houA	juvenile	06/13/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
4876	MF04876	juvenile	06/13/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	HM021099
4905		male	03/31/2001	30.20198	-97.20898	USA	Texas	Bastrop	BAN10p	GLR pond 11	2, 4	
4907		female	04/16/2001	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	2, 4	
4908		male	03/31/2001	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
4909		male	03/31/2001	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
4910		male	03/31/2001	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	2, 4	
4911		male	03/31/2001	30.20198	-97.20898	USA	Texas	Bastrop	BAN10p	GLR pond 11	2, 4	
4912		male	03/31/2001	30.20198	-97.20898	USA	Texas	Bastrop	BAN10p	GLR pond 11	2, 4	
4914		male	03/31/2001	30.20198	-97.20898	USA	Texas	Bastrop	BAN10p	GLR pond 11	2, 4	
4978		female?	03/30/2001	30.31281	-97.15247	USA	Texas	Lee	LEE01s	CR-333, 2.7 mi S jct CR-331 & CR-333	2, 4	
5408		juvenile	07/07/2001			USA	Texas	Bastrop	BAN04p	GLR 4-E	2, 4	
5706	houA	tadpole	04/14/2002	30.21427	-97.23254	USA	Texas	Bastrop	BAN05p	GLR pond 6	2, 4	
5707	MF05707	tadpole	04/14/2002	30.21427	-97.23254	USA	Texas	Bastrop	BAN05p	GLR pond 6	2, 4	HM021103
5720	houA	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5727	houD	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5728	houD	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5729	houD	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4, 5	HM021033
5756	houB	tadpole	04/17/2002	30.32482	-97.16896	USA	Texas	Lee	LEE02p	Durham pond 1	2, 4	
5757	houB	tadpole	04/17/2002	30.32482	-97.16896	USA	Texas	Lee	LEE02p	Durham pond 1	2, 4	
5758	houD	tadpole	04/17/2002	30.32482	-97.16896	USA	Texas	Lee	LEE02p	Durham pond 1	2, 4	
5759	houB	tadpole	04/17/2002	30.32482	-97.16896	USA	Texas	Lee	LEE02p	Durham pond 1	2, 4	
5760	houB	tadpole	04/17/2002	30.32482	-97.16896	USA	Texas	Lee	LEE02p	Durham pond 1	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo houstonensis* continued**												
5761	houD	tadpole	04/17/2002	30.32482	-97.16896	USA	Texas	Lee	LEE02p	Durham pond 1	2, 4	
7927	houA	juvenile	06/24/2002			USA	Texas	Bastrop	BAN02p	GLR refugia NW of pond 2 (#3)	2, 4	
7928	houA	juvenile	04/19/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
7929	houE	juvenile	04/19/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	HM021106
7930	houE	juvenile	04/19/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
7931	houE	juvenile	04/19/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
7932	houA	juvenile	04/19/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
7934	houA	juvenile	04/19/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
7935	houE	juvenile	04/19/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	HM021051
8239		male	02/14/2003	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5A	2, 4	
8472		male	01/25/2004			USA	Texas	Bastrop	BAN04p	GLR B-F4	2, 4	
8904		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	HM021039
8905		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8906		male	04/08/2002	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	2, 4	
8907		male	03/20/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8908		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8909		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8912		male	03/09/2002	30.21658	-97.24097	USA	Texas	Bastrop	BAN14t	GLR 3-1	2, 4	
8913		male	03/20/2002	30.21528	-97.23139	USA	Texas	Bastrop	BAN17t	GLR 6-S	2, 4	
8914		male	03/13/2002	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
8916		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8918		male	03/14/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8919		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8921		male	03/03/2002	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	2, 4	
8922		male	03/07/2002	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1-1	2, 4	
8923		male	03/13/2002	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
8924		female	12/16/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8925		female	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	HM021055
8926		male	03/13/2002	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
8927		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	HM021056
8928		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
8929		male	03/14/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9069		male	03/07/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9071		male	03/07/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9072		male	03/07/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9073		female	03/07/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9074		male	03/07/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9322	houA	juvenile	04/20/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9323	houA	juvenile	04/20/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9324		juvenile	04/20/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9325	houA	juvenile	04/20/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9350		female	03/12/2003	30.21029	-97.24548	USA	Texas	Bastrop	BAN26t	GLR trap K-9	2, 4	
9351	MF09351	juvenile	11/18/2003	30.19989	-97.2172	USA	Texas	Bastrop	BAN20t	GLR 12-1	2, 4	HM021109
9354		male	03/12/2003	30.16953	-97.24165	USA	Texas	Bastrop	BAN01p	1441 & Old Fire Tower Rd	2, 4	
9752		female	02/24/2004	30.21029	-97.24548	USA	Texas	Bastrop	BAN26t	GLR trap K-3	2, 4	
9754		male	02/24/2004	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1-1	2, 4	
9766		female	02/24/2004			USA	Texas	Bastrop	BAN05p	GLR 5-S	2, 4	
9770		female	03/16/2004			USA	Texas	Bastrop	BAN05p	GLR 5-F6	2, 4	
16651		male	02/18/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
16652		male	02/18/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
16655		male	02/18/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
16661		male	04/07/2002	30.21427	-97.23254	USA	Texas	Bastrop	BAN05p	GLR pond 6	2, 4	
16662		male	04/07/2002	30.21427	-97.23254	USA	Texas	Bastrop	BAN05p	GLR pond 6	2, 4	
16664		male	04/07/2002	30.21427	-97.23254	USA	Texas	Bastrop	BAN05p	GLR pond 6	2, 4	
16666		male	03/27/2002	30.20986	-97.24003	USA	Texas	Bastrop	BAN25t	GLR C-F4	2, 4	
16667		male	03/25/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
16679		male	05/28/2002	30.21528	-97.23139	USA	Texas	Bastrop	BAN17t	GLR 6-1	2, 4	
16709		male	03/27/2003	30.20953	-97.24197	USA	Texas	Bastrop	BAN24t	GLR B-1	2, 4	
16710		male	03/27/2003	30.21647	-97.24178	USA	Texas	Bastrop	BAN13t	GLR 2-1	2, 4	
16711		male	03/27/2003	30.19981	-97.21703	USA	Texas	Bastrop	BAN21t	GLR 12-2	2, 4	
16715		male	02/13/2003			USA	Texas	Bastrop	BAN02p	GLR 2-W	2, 4	
16718		male	02/13/2003			USA	Texas	Bastrop	BAN05p	GLR 5-F6	2, 4	
16866		male	02/13/2003			USA	Texas	Bastrop	BAN02p	GLR 2-W	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo houstonensis* continued**												
16988		female	05/02/2004	30.21647	-97.24178	USA	Texas	Bastrop	BAN13t	GLR 2-1	2, 4	
16989		female	05/08/2004	30.21647	-97.24178	USA	Texas	Bastrop	BAN13t	GLR 2-1	2, 4	
17003		male	04/04/2004	30.2106	-97.24802	USA	Texas	Bastrop	BAN03s	near GLR pond 3	2, 4	
17010		female	03/03/2004	30.20008	-97.22266	USA	Texas	Bastrop	BAN18t	GLR 10-1	2, 4	
17011		male	03/12/2004	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17012		male	03/12/2004	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17019		male	03/12/2004	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17020		female	03/15/2004	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17024		male	03/15/2004	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17025		female	03/14/2004			USA	Texas	Bastrop	BAN02p	GLR A-W	2, 4	
17026		male	03/03/2004	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	2, 4	
17027		male	03/03/2004	30.21436	-97.23325	USA	Texas	Bastrop	BAN16t	GLR 5-1	2, 4	
17028		male	03/03/2004	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
17029		male	03/03/2004	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	2, 4	
17030		male	03/03/2004	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	2, 4	
17031		male	03/03/2004	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
17032		male	02/23/2004	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
17033		male	02/23/2004	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
17037		male	02/20/2004	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	2, 4	
17038	houA	juvenile	04/03/2003			USA	Texas	Bastrop	BAN02p	GLR 2-F2	2, 4	
17045		male	01/16/2004			USA	Texas	Bastrop	BAN02p	GLR 2-F4	2, 4	
17054		male	01/15/2004			USA	Texas	Bastrop	BAN06p	GLR 7-S	2, 4	
17055		male	03/14/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17071		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17075		male	04/09/2002			USA	Texas	Bastrop	BAN02p	GLR 1-N	2, 4	
17076		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17077		female	03/01/2002	30.19575	-97.21494	USA	Texas	Bastrop	BAN22t	GLR 14-5	2, 4	
17080		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17081		male	03/08/2002	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	2, 4	
17082		female	03/14/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17086		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17087		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17088		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17089		male	03/20/2002			USA	Texas	Bastrop	BAN02p	GLR 2-W	2, 4	
17092		male	02/19/2002			USA	Texas	Bastrop	BAN02p	GLR 1-E	2, 4	
17093		male	04/01/2002	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	2, 4	
17094		male	03/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
17095		male	03/09/2002	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	2, 4	
17096		male	03/13/2002	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
17097		male	03/20/2002	30.21528	-97.23139	USA	Texas	Bastrop	BAN17t	GLR 6-1	2, 4	
17098		male	03/09/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17101		male	03/09/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17102		male	03/09/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4, 5	HM021046
17103		male	03/09/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17104		male	03/12/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17105		male	??/??/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17107		male	04/11/2002	30.21436	-97.23325	USA	Texas	Bastrop	BAN16t	GLR 5-1	2, 4	
17283		male	03/21/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17284		male	03/21/2005	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
17285		male	03/21/2005	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
17286		male	03/21/2005	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
17287		male	04/02/2003	30.2056	-97.23424	USA	Texas	Bastrop	BAN07p	GLR pond 8	2, 4	
17289		male	04/07/2003	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1-1	2, 4	
17290		female	04/05/2003			USA	Texas	Bastrop	BAN02p	GLR 2-F2	2, 4	
17291		male	04/02/2003	30.2056	-97.23424	USA	Texas	Bastrop	BAN07p	GLR pond 8	2, 4	
17292		male	04/01/2003	30.2056	-97.23424	USA	Texas	Bastrop	BAN07p	GLR pond 8	2, 4	
17293		male	03/27/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17294		male	03/03/2003	30.21029	-97.24548	USA	Texas	Bastrop	BAN26t	GLR trap K-2	2, 4	
17301		male	03/18/2003	30.21029	-97.24548	USA	Texas	Bastrop	BAN26t	GLR trap K-1	2, 4	
17302		male	04/01/2003	30.2056	-97.23424	USA	Texas	Bastrop	BAN07p	GLR pond 8	2, 4	
17303		male	04/06/2003	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
17310		male	02/15/2005	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
17311		male	02/14/2005	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
17312		male	02/15/2005	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo houstonensis* continued**												
17313		male	02/15/2005	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
17314		male	02/15/2005	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
17315		male	02/16/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17316		male	02/16/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17317		male	02/16/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17318		male	02/15/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17319		male	02/16/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17320		male	02/15/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17321		male	02/15/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17322		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17323		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17324		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17325		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17326		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17327		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17328		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	HM021062
17329		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17330		male	02/22/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17331		male	03/01/2005	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
17332		male	03/05/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17333		male	03/06/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17334		male	03/06/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17335		male	03/05/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17336		male	03/06/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17337		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17338		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17339		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17340		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17341		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17342		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17343		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17344		female	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17345		male	03/05/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17346		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17347		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17348		male	03/07/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17349		male	03/06/2005	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
17350		male	03/20/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17351		male	03/20/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17352		male	03/20/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17353		male	03/20/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17354		male	03/20/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4, 5	
17357		male	03/20/2005	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
17358		male	03/20/2005	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
17359		male	03/20/2005	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
17360		male	03/20/2005	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
17361		male	03/20/2005	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
17362		male	03/20/2005	30.21235	-97.23	USA	Texas	Bastrop	BAN06p	GLR pond 7	2, 4	
19761	houB	juvenile	06/15/2005	30.10428	-97.2682	USA	Texas	Bastrop	BAS10t	BSP pond 10 C1, Bucket #6	2, 4, 5	HM021025
19773	houB	juvenile	07/13/2005	30.12065	-97.26009	USA	Texas	Bastrop	BAS12t	BSP pond 1 T1, Bucket # 7	2, 4	
19774	houH	juvenile	07/13/2005			USA	Texas	Bastrop	BAS12t	BSP pond 1 T1, On Trap Line	2, 4	HM021111
19775	houC	juvenile	07/10/2005			USA	Texas	Bastrop	BAS11t	BSP Harmon Rd, On Trap Line	2, 4	
19782	houB	juvenile	07/15/2005	30.12069	-97.26204	USA	Texas	Bastrop	BAS13t	BSP pond 1 T1, Bucket # 3	2, 4	
19784	houG	juvenile	07/15/2005	30.10094	-97.25169	USA	Texas	Bastrop	BAS11t	BSP Harmon Rd, Bucket # 1	2, 4	
19785	houC	juvenile	07/15/2005			USA	Texas	Bastrop	BAS11t	BSP Harmon Rd, Trap # 8	2, 4	
19977	houC	male	03/28/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
19978		male	03/28/2006			USA	Texas	Bastrop	BAS18p	Jim Small pond 6	2, 4	
19979		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19980		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19981		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo houstonensis* continued**												
19982		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19983		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19984	houC	male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19985		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19986	houA	male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19987		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19988		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19989		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19990	houE	male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19991	houA	male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19992		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
19995		male	03/30/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
19996	houB	male	03/30/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
19997	houB	male	03/30/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
19998		male	03/30/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
19999		male	03/30/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
20000	houB	male	03/30/2006	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	2, 4	
20001	houB	male	03/30/2006	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	2, 4	
20002		male	03/30/2006	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	2, 4	
20004		male	03/30/2006	30.14108	-97.24349	USA	Texas	Bastrop	BAS16p	Jim Small pond 3	2, 4	
20005		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20006		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20007		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20008		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20009		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20011	houB	male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20012		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20013		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20014		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
20024		male	03/30/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
20025		male	03/30/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
20026		male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
20027		male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
20028	houA	male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
20029		male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
20030	houA	male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
20031		male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
20032	houD	male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	HM021112
20033		male	03/30/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
20040	houA	male	03/30/2006	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
20041	houB	male	03/30/2006	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
20042	houB	male	03/30/2006	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
20043	houB	male	03/30/2006	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
20044	houB	male	03/30/2006	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
20045		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20046		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20047		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20048	houB	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20049	houB	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20050		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20051		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20052	houA	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20053		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20054		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20055		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20056	houC	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20058		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20060		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20061	houB	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20062	houC	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20063		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20064	houC	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20065		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	
20066		male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo houstonensis* continued**												
20073	MF20073	male	03/29/2006	31.0775	-96.19334	USA	Texas	Leon	LEOp	Hilltop Lakes	2, 4	HM021113
20196		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
20197		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
20363		male	04/23/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
21331	houA	juvenile	06/14/2003	30.21586	-97.23928	USA	Texas	Bastrop	BAN23t	GLR A	2, 4	
21334		male	02/22/2005	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	2, 4	
21972		male	02/22/2005	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	2, 4	
22252	houC	male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22253		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22254		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22255	houC	male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22256		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22257		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22258		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22259	houB	male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22260		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22261	houC	male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22262		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22263		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22264	houC	male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22265		male	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22266		female	02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22267			02/20/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22268	houC	male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22269		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22270		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22271		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22272		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22273		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22274		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22275	houB	male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22276		female	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22277		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22278		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22279			02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22280		male	02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22281			02/21/2007	30.14194	-97.26205	USA	Texas	Bastrop	BAS04p	BBHQ pond 3	2, 4	
22282		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22284		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22285		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22286	houC	male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22287		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22289		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22290		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22292		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22293		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22294	houH	male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	2, 4	
22295	houB	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22296	houB	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22297	houC	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22298		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4, 5	HM021088
22299		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22300	houA	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22301	houA	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22302		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22303		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22304		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22305		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22306		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22307	houC	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22308		male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22309	houC	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22310	houC	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22311	houB	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo houstonensis* continued**												
22312	houB	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22313	houB	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22314	houB	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22315	houC	male	02/21/2007	30.0957	-97.23859	USA	Texas	Bastrop	BAS07p	BSP pond 8	2, 4	
22316	houC	male	02/28/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	2, 4	
22317	houB	male	02/28/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	2, 4	
22318	houC	male	02/28/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	2, 4	
22319		male	02/28/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	2, 4	
22320	houH	male	02/28/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	2, 4	
22324	houA	male	02/27/2007	30.255	-97.22787	USA	Texas	Bastrop	BAN29s	E HWY 290 at Sandy Creek	2, 4	
22325	houA	male	02/27/2007	30.255	-97.22787	USA	Texas	Bastrop	BAN29s	E HWY 290 at Sandy Creek	2, 4	
22326	houA	male	02/27/2007	30.255	-97.22787	USA	Texas	Bastrop	BAN29s	E HWY 290 at Sandy Creek	2, 4	
22327		male	02/27/2007	30.255	-97.22787	USA	Texas	Bastrop	BAN29s	E HWY 290 at Sandy Creek	2, 4	
22328		male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22329		female	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22330		female	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22331	houA	male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22332	houA	male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22333	houA	male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22334		male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22335		male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22336		male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22337	houA	male	02/28/2007	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	2, 4	
22338		male	02/28/2007	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
22339		male	02/28/2007	30.19918	-97.22197	USA	Texas	Bastrop	BAN08p	GLR pond 9	2, 4	
22347	houC	male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22348	houC	male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22349		male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22350		male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22351	houC	male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22352		male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22353	houC	male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22354		male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22355		male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22356	houB	male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22357		female?	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22359		male	03/14/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	2, 4	
22360		male	03/14/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	2, 4	
22361	houC	male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22362	houA	male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22363		male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22364	houC	male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22365	houC	male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22366		male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22367	houB	male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22368		male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22370		male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22371	houE	male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	2, 4	
22389		male	03/20/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	2, 4, 5	HM021043
22390	houB	male	03/25/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	2, 4	
22396	houF	male	03/12/2007	30.7135	-96.74612	USA	Texas	Milam	MILs	CR-342	2, 4	
22397	houB	male	03/12/2007	30.7135	-96.74612	USA	Texas	Milam	MILs	CR-342	2, 4	
22398	houB	male	03/12/2007	30.7135	-96.74612	USA	Texas	Milam	MILs	CR-342	2, 4	
22418	houA	male	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	2, 4, 5	HM021071, HM021075, HM021079
22423	houA	male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	2, 4	
22424	houC	male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	2, 4	
22425		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	2, 4	
22441	houB	male	03/27/2007	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
22442	houB	male	03/27/2007	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	2, 4	
22443	houC	male	03/27/2007	30.13959	-97.26137	USA	Texas	Bastrop	BAS05s	BBHQ path to BBHQ pond 3	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo houstonensis* continued**												
22444	houB	male	03/27/2007	30.14018	-97.2706	USA	Texas	Bastrop	BAS02p	BBHQ pond 2	2, 4	
22445	houC	male	03/27/2007	30.13874	-97.26881	USA	Texas	Bastrop	BAS03s	BBHQ creek upstream of pond 2	2, 4	
22446		male	03/27/2007	30.13874	-97.26881	USA	Texas	Bastrop	BAS03s	BBHQ creek upstream of pond 2	2, 4	
22447		male	03/28/2007	30.14018	-97.2706	USA	Texas	Bastrop	BAS02p	BBHQ pond 2	2, 4	
22448	houC	male	03/28/2007	30.14018	-97.2706	USA	Texas	Bastrop	BAS02p	BBHQ pond 2	2, 4	
22449	houB	male	03/28/2007	30.14018	-97.2706	USA	Texas	Bastrop	BAS02p	BBHQ pond 2	2, 4	
22451	houF	male	03/27/2007	29.84165	-96.4889	USA	Texas	Colorado	COLs	CR-52, near jct w/ Warsehak Schuette Rd	2, 4, 5	HM021082, HM021092
22452	houF	male	03/27/2007	29.84165	-96.4889	USA	Texas	Colorado	COLs	CR-52, near jct w/ Warsehak Schuette Rd	2, 4	
22453	houF	male	03/27/2007	29.84165	-96.4889	USA	Texas	Colorado	COLs	CR-52, near jct w/ Warsehak Schuette Rd	2, 4	
22470	houE	male	03/12/2007	30.17795	-97.2338	USA	Texas	Bastrop	BAN11p	GLR pond 15	2, 4	
22471		male	03/12/2007	30.17795	-97.2338	USA	Texas	Bastrop	BAN11p	GLR pond 15	2, 4	
22483	houA	male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	2, 4	
22529	houB	female	04/03/2007	30.7135	-96.74612	USA	Texas	Milam	MILs	CR-342	2, 4	
26267	houF	male	04/02/2008	29.87789	-96.35294	USA	Texas	Austin	AUS03p	TCW Pond	2, 4, 5	HM021030, HM021116
26269	houB	male	04/02/2008	29.87789	-96.35294	USA	Texas	Austin	AUS03p	TCW Pond	2, 4	HM021117
26271	houB	male	04/10/2008	29.87246	-96.36386	USA	Texas	Austin	AUS01p	500 m E of jct FM-1094 & Hinkel Rd	2, 4	
26416	houB	male	04/26/2008	29.88395	-96.36161	USA	Texas	Austin	AUS02s	Hall Rd at jct of Hall Rd & Hinkel Rd	2, 4	
Bufo nebulifer												
2490		male	06/13/1998	29.80993	-99.57344	USA	Texas	Bandera	BND03s	Lost Maples Natural Area, 5 rd mi N of Vanderpool, trap 1 F2	3, 4	
2893	nebD	male	08/07/1998	29.74234	-99.08241	USA	Texas	Bandera	BND02s	6.6 rd mi S of Jct FM2828 & FM3240 on 3240	3, 4	HM021119
2894	nebC	female	08/07/1998	29.69726	-99.05061	USA	Texas	Bandera	BND01s	1.1 rd mi S of jct Hwy 173 & Whartons Dock Rd	3, 4	HM021120
3360	nebD	tadpole	04/24/2000	30.37764	-97.24774	USA	Texas	Lee	LEE10p	F4 pond 3	3, 4	
3361	nebD	tadpole	04/24/2000	30.37764	-97.24774	USA	Texas	Lee	LEE10p	F4 pond 3	3, 4	
3362	nebA	tadpole	04/24/2000	30.37143	-97.24534	USA	Texas	Lee	LEE09p	F4 pond 1	3, 4	
3370	nebD	tadpole	04/24/2000	30.37764	-97.24774	USA	Texas	Lee	LEE10p	F4 pond 3	3, 4	
3385	nebA	tadpole	04/24/2000	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	3, 4	
3402	nebC	tadpole	04/24/2000	30.34278	-97.18426	USA	Texas	Lee	LEE15p	McManus Pond 1	3, 4	
3403	nebA	tadpole	04/24/2000	30.34278	-97.18426	USA	Texas	Lee	LEE15p	McManus Pond 1	3, 4	
3426	nebA	tadpole	04/24/2000	30.34596	-97.18395	USA	Texas	Lee	LEE16p	McManus Pond 2	3, 4	
3427	nebA	tadpole	04/24/2000	30.34596	-97.18395	USA	Texas	Lee	LEE16p	McManus Pond 2	3, 4	
3428	nebA	tadpole	04/24/2000	30.34596	-97.18395	USA	Texas	Lee	LEE16p	McManus Pond 2	3, 4	
3432	nebA	tadpole	??/??/2000	30.31756	-97.26781	USA	Texas	Bastrop	BAN33p	B3 pond 2	3, 4	
3433	nebA	tadpole	??/??/2000	30.31756	-97.26781	USA	Texas	Bastrop	BAN33p	B3 pond 2	3, 4	
3490	nebA		04/14/2000	30.54669	-96.34524	USA	Texas	Brazos	BRA01s	Blue Ridge Drive	3, 4	
3491	nebA		04/14/2000	30.54669	-96.34524	USA	Texas	Brazos	BRA01s	Blue Ridge Drive	3, 4	
3493	nebA		??/??/2000	29.79165	-97.93415	USA	Texas	Guadalupe	GUA01p	Ranch, House Pond	3, 4, 5	HM021035
3497	nebA		??/??/2000	29.79165	-97.93415	USA	Texas	Guadalupe	GUA01p	Ranch, House Pond	3, 4	
3511	nebA	tadpole	04/24/2000	30.33328	-97.18054	USA	Texas	Lee	LEE07p	AC Willis pond 3	3, 4	
3512	nebA	tadpole	04/24/2000	30.33328	-97.18054	USA	Texas	Lee	LEE07p	AC Willis pond 3	3, 4	
3513	nebA	tadpole	04/24/2000	30.33328	-97.18054	USA	Texas	Lee	LEE07p	AC Willis pond 3	3, 4, 5	HM021067, HM021072, HM021076
3514	nebB	tadpole	04/24/2000	30.32586	-97.17738	USA	Texas	Lee	LEE05p	AC Willis pond 1	3, 4	
3515	nebA	tadpole	04/24/2000	30.32586	-97.17738	USA	Texas	Lee	LEE05p	AC Willis pond 1	3, 4	
3516	nebB	tadpole	04/24/2000	30.32586	-97.17738	USA	Texas	Lee	LEE05p	AC Willis pond 1	3, 4	
3517	nebA	tadpole	04/24/2000	30.32586	-97.17738	USA	Texas	Lee	LEE05p	AC Willis pond 1	3, 4	
3518	nebA	tadpole	04/24/2000	30.32586	-97.17738	USA	Texas	Lee	LEE05p	AC Willis pond 1	3, 4	
3519	nebA	tadpole	04/24/2000	30.32586	-97.17738	USA	Texas	Lee	LEE05p	AC Willis pond 1	3, 4	
3520	nebA	tadpole	04/24/2000	30.33374	-97.18208	USA	Texas	Lee	LEE06p	AC Willis pond 2	3, 4	
3521	nebA	tadpole	04/24/2000	30.33374	-97.18208	USA	Texas	Lee	LEE06p	AC Willis pond 2	3, 4	
3522	nebA	tadpole	04/24/2000	30.33374	-97.18208	USA	Texas	Lee	LEE06p	AC Willis pond 2	3, 4	
3641	nebA		02/24/2001	30.2056	-97.23424	USA	Texas	Bastrop	BAN07p	GLR pond 8	3, 4	
3652	nebA		02/24/2001	30.20997	-97.24821	USA	Texas	Bastrop	BAN32p	GLR pond 3	3, 4	
3653	nebA		02/24/2001	30.20997	-97.24821	USA	Texas	Bastrop	BAN32p	GLR pond 3	3, 4	
3654	nebA		02/24/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
3657		female	06/10/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
3661		male	06/15/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3663		female	06/21/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3667		female	06/12/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3668		female	06/20/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3675		male	06/21/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3678	nebA		06/20/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3680		female	06/10/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3683		male	06/20/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3700		male	06/12/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3740		unknown	06/20/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3764	nebA		06/03/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3775	nebA	male	06/10/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3797	nebA	male	06/12/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3825	nebA	male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3826	nebB	male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3827		male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3828		male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3829		male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3830		male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3831		male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3832		male	04/04/2001	30.32186	-97.12761	USA	Texas	Lee	LEE04p	1.3 mi N of Lee Co. line on CR-333 at rock quarry	3, 4	
3981	nebC		05/12/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
3988	nebA		05/12/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	3, 4	
4429	nebA	tadpole	??/??/2001	30.30978	-97.16222	USA	Texas	Bastrop	BAN41p	Salamander Pond CR-333	3, 4	
4430	nebA	tadpole	??/??/2001	30.30978	-97.16222	USA	Texas	Bastrop	BAN41p	Salamander Pond CR-333	3, 4	
4431	nebA	tadpole	??/??/2001	30.30978	-97.16222	USA	Texas	Bastrop	BAN41p	Salamander Pond CR-333	3, 4	
4442		tadpole	04/16/2001	30.36224	-97.25602	USA	Texas	Bastrop	BAN31p	E3 pond 6	3, 4	
4443	nebA	tadpole	04/16/2001	30.36224	-97.25602	USA	Texas	Bastrop	BAN31p	E3 pond 6	3, 4	
4444		tadpole	04/16/2001	30.36224	-97.25602	USA	Texas	Bastrop	BAN31p	E3 pond 6	3, 4	
4447	nebB	tadpole	04/16/2001	30.38786	-97.20094	USA	Texas	Lee	LEE12p	G6 pond 9	3, 4	
4449	nebB	tadpole	04/16/2001	30.38786	-97.20094	USA	Texas	Lee	LEE12p	G6 pond 9	3, 4	
4450	nebB	tadpole	04/16/2001	30.38786	-97.20094	USA	Texas	Lee	LEE12p	G6 pond 9	3, 4	
4452	nebB	tadpole	04/16/2001	30.41608	-97.18367	USA	Texas	Lee	LEE13p	I7 pond 10	3, 4	
4453		tadpole	04/16/2001	30.41608	-97.18367	USA	Texas	Lee	LEE13p	I7 pond 10	3, 4	
4502	nebB	tadpole	04/20/2001	30.31743	-97.12102	USA	Texas	Lee	LEE17p	Quarry small	3, 4	
4506	nebA	tadpole	04/20/2001	30.31743	-97.12102	USA	Texas	Lee	LEE17p	Quarry small	3, 4	
4507	nebB	tadpole	04/20/2001	30.31743	-97.12102	USA	Texas	Lee	LEE17p	Quarry small	3, 4	
4508	nebB	tadpole	04/20/2001	30.31743	-97.12102	USA	Texas	Lee	LEE17p	Quarry small	3, 4	
4509	nebC	tadpole	04/20/2001	30.37191	-97.21108	USA	Texas	Lee	LEE11p	F6 Pond 4	3, 4	
4511		tadpole	04/20/2001	30.37191	-97.21108	USA	Texas	Lee	LEE11p	F6 Pond 4	3, 4	
4524	nebA	tadpole	04/20/2001	30.37191	-97.21108	USA	Texas	Lee	LEE11p	F6 Pond 4	3, 4	
4730		male	04/16/2001	30.19936	-97.21639	USA	Texas	Bastrop	BAN35t	GLR 12-4	3, 4	
4737		female	04/16/2001	30.21036	-97.23828	USA	Texas	Bastrop	BAN15t	GLR 4	3, 4	
4738		male	04/17/2001	30.20014	-97.22173	USA	Texas	Bastrop	BAN34t	GLR 10-3	3, 4	
4823		male	04/01/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1	4	
4828		female	04/03/2001	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	3, 4	
4829		male	04/05/2001	30.2002	-97.22236	USA	Texas	Bastrop	BAN19t	GLR 10-2	3, 4	
4831		male	04/05/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1	3, 4	
4832		male	04/05/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1	3, 4	
4833		male	04/05/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1	3, 4	
4834		male	04/05/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1	3, 4	
4835		male	04/05/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1	3, 4	
4836		male	04/05/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1	3, 4	
4870	nebA	juvenile	05/27/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
4872	nebA	juvenile	06/10/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
4886	nebA	juvenile	06/17/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
4887	nebA	juvenile	06/16/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
4980			03/30/2001	30.31102	-97.15821	USA	Texas	Lee	LEE08s	CR-333, Lee Co. line	3, 4	
4981	nebA	male	03/30/2001	30.31102	-97.15821	USA	Texas	Lee	LEE08s	CR-333, Lee Co. line	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
4982		female	03/30/2001	30.31102	-97.15821	USA	Texas	Lee	LEE08s	CR-333, Lee Co. line	3, 4	
4983		male	03/30/2001	30.31102	-97.15821	USA	Texas	Lee	LEE08s	CR-333, Lee Co. line	3, 4	
4985		male	03/30/2001	30.31102	-97.15821	USA	Texas	Lee	LEE08s	CR-333, Lee Co. line	3, 4	
4986	nebD		03/30/2001	30.31102	-97.15821	USA	Texas	Lee	LEE08s	CR-333, Lee Co. line	3, 4	
5379	nebA	juvenile	07/14/2001			USA	Texas	Bastrop	BAN02p	GLR 1-N	3, 4	
7372		male	06/21/2002	30.4317	-96.7559	USA	Texas	Burleson	BUR13s	Deanville	3, 4	
8525		male	03/29/2004			USA	Texas	Bastrop	BAN02p	GLR 1-F5	3, 4	
8567		male	04/25/2004			USA	Texas	Bastrop	BAN04p	GLR C-F5	3, 4	
8580		female	04/25/2004	30.21658	-97.24097	USA	Texas	Bastrop	BAN14t	GLR 3-1	3, 4	
8594		male	05/02/2004			USA	Texas	Bastrop	BAN02p	GLR 1-F1	3, 4	
8595		female	05/02/2004			USA	Texas	Bastrop	BAN02p	GLR 3-F5	3, 4	
8596		female	05/02/2004	30.21586	-97.23928	USA	Texas	Bastrop	BAN23t	GLR A-1	3, 4	
8613		male	05/23/2004			USA	Texas	Bastrop	BAN02p	GLR 2-F1	3, 4	
8621		male	06/08/2004	30.21658	-97.24097	USA	Texas	Bastrop	BAN14t	GLR 3-1	3, 4	
8627		female	06/09/2004			USA	Texas	Bastrop	BAN02p	GLR 3-F4	3, 4	
8628		male	06/09/2004	30.21658	-97.24097	USA	Texas	Bastrop	BAN14t	GLR 3-1	3, 4	
8638		female	06/11/2004			USA	Texas	Bastrop	BAN02p	GLR 2-F5	3, 4	
8643	nebA	male	06/15/2004	30.3146	-97.7652	USA	Texas	Travis	TRA01t	Camp Mabry, funnel trap	3, 4	
8644	nebA	female	06/15/2004	30.3146	-97.7652	USA	Texas	Travis	TRA01t	Camp Mabry, funnel trap	3, 4	
8652		female	06/26/2004			USA	Texas	Guadalupe	GUA02t	Ranch 5-1	3, 4	
8653		female	06/26/2004			USA	Texas	Guadalupe	GUA02t	Ranch 2-F2	3, 4	
8660	nebA	female	06/29/2004			USA	Texas	Guadalupe	GUA02t	Ranch 1-1	3, 4	
8663	nebA	juvenile	07/01/2004			USA	Texas	Guadalupe	GUA02t	Ranch 2-2	3, 4, 5	HM021031
8665	nebA	juvenile	07/02/2004			USA	Texas	Guadalupe	GUA02t	Ranch 2-2	3, 4	
8686	nebA	juvenile	08/05/2004	27.10226	-97.44064	USA	Texas	Kenedy	KEN01s	Sand Castle Fishing Cabin, 30 sea mi S of Bird Island	3, 4	
8688	nebA	female	08/05/2004	27.10226	-97.44064	USA	Texas	Kenedy	KEN01s	Sand Castle Fishing Cabin, 30 sea mi S of Bird Island	3, 4	
8689	nebA	juvenile	08/05/2004	27.10226	-97.44064	USA	Texas	Kenedy	KEN01s	Sand Castle Fishing Cabin, 30 sea mi S of Bird Island	3, 4	
8775	nebA	juvenile	05/15/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8776	nebC	juvenile	05/16/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8777	nebC	juvenile	05/16/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8782	nebA	juvenile	05/16/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8783	nebC	juvenile	05/18/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8784	nebC	juvenile	05/18/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8785	nebC	juvenile	05/18/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8786	nebC	juvenile	05/18/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
8787	nebC	juvenile	05/18/2003	30.18113	-97.212073	USA	Texas	Bastrop	BAN40s	Ponderosa Dr	3, 4	
9536		male	07/09/2004	23.40495	-99.36947	Mexico	Tamaulipas		MEX05s	Jaumave	3, 4	
9537		male	07/09/2004	23.40495	-99.36947	Mexico	Tamaulipas		MEX05s	Jaumave	3, 4	
9538		male	07/09/2004	23.38668	-99.39328	Mexico	Tamaulipas		MEX03s	2.4 km SW of Jaumave	3, 4	
9539		male	07/09/2004	23.40495	-99.36947	Mexico	Tamaulipas		MEX05s	Jaumave	3, 4	
9595		female	07/14/2004	24.99848	-98.30267	Mexico	Tamaulipas		MEX04s	22 km NW of San Fernando	3, 4	
9820	nebA	female	04/10/2004	30.43611	-101.78592	USA	Texas	Terrell	TER01s	Oasis Ranch	3, 4, 5	HM021028
9833	nebA	juvenile	04/10/2004	30.43611	-101.78592	USA	Texas	Terrell	TER01s	Oasis Ranch	3, 4	
9864	nebA	juvenile	04/10/2004	30.43611	-101.78592	USA	Texas	Terrell	TER01s	Oasis Ranch	3, 4	
9879	nebA		05/23/2003	30.00302	-101.1714	USA	Texas	Val Verde	VAL01s	Hwy 63, 38 km N of jct w/US90	3, 4	
9950		female	05/19/2004	30.21658	-97.24097	USA	Texas	Bastrop	BAN14t	GLR 3-1	3, 4, 5	HM021057
9963		male	05/18/2004			USA	Texas	Bastrop	BAN02p	GLR 3-F6	3, 4	
9983	nebE		02/28/2004			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	HM021123
9985	nebA	juvenile	02/28/2004			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	
9990	nebA	juvenile	02/28/2004			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	
9994	nebG		02/29/2004			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	HM021124
10019	nebA		02/29/2004			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	
16911	nebB		10/23/2004	23.80923	-98.26467	Mexico	Tamaulipas		MEX06s	Municipality of Casas	3, 4	HM021125
16984		male	04/24/2004			USA	Texas	Bastrop	BAN04p	GLR C-F5	3, 4	
16991		male	05/12/2004	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
16992		female	04/24/2004	30.21528	-97.23139	USA	Texas	Bastrop	BAN17t	GLR 6-1	3, 4	
16997		female	04/29/2004	30.19611	-97.21547	USA	Texas	Bastrop	BAN36t	GLR 14-3	3, 4	
17002		female	04/29/2004	30.20953	-97.24197	USA	Texas	Bastrop	BAN24t	GLR B-1	3, 4	
17057	nebC		04/08/2002	30.20014	-97.22173	USA	Texas	Bastrop	BAN34t	GLR 10-3	3, 4	
17058		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
17061		female	08/15/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1-1	3, 4	
17064		juvenile	09/16/2001	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1-1	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
17065	nebA		04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
17068	nebA	male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
17074	nebA	male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
17079		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
17306		female	05/11/2003			USA	Texas	Bastrop	BAN37t	GLR 18-2	3, 4	
17567		male	04/08/2005	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
17570		male	04/08/2005	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
17571		male	04/08/2005	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
17572		male	04/08/2005	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
19729		male	05/05/2005	29.79252	-97.9366	USA	Texas	Guadalupe	GUA03t	Ranch Funnel 3-F2	3, 4	
19993		male	03/29/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
19994	nebA	male	03/30/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20003		male	03/30/2006	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
20015		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	3, 4	
20074	nebF	male	03/30/2006	31.0775	-96.19334	USA	Texas	Leon	LEOp	Hilltop Lakes	3, 4	HM021126
20091		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20093	nebA	male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20094	nebA	male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20095		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20096		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20097		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20098		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20099		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20100		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20101		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20102		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20103		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20104		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20105		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20106		male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20107	nebA	male	04/22/2006	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
20108		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20109	nebA	male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20110	nebA	male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20111		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20112		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20113		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20114		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20115		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20116		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20117		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	4	
20118		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20119		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20120		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20121		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20122		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20123		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20124		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20125		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20126		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20127		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20128		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20129		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20130		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20131		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20132		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20133		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20134		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20135		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20136		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20137		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20138		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20139		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20140		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
20132		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20133		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20134		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20135		male	04/22/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	3, 4, 5	
20136		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20137		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20138		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20139		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20140		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20141		male	04/23/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20142		male	04/23/2006	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
20143		male	04/23/2006	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
20144	nebA	male	04/23/2006	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
20145		male	04/23/2006	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
20146		male	04/23/2006	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
20147		male	04/23/2006	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
20148		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20149		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20150		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20151		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20152		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20153		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20154		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20155		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20156		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20157		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20158		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20159		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20160		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20161		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20162		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20163		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20164		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20165		female	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20166		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20167		male	04/21/2006	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	3, 4	
20174	nebA	female	04/24/2006	29.88442	-97.93918	USA	Texas	Hays	HAY01s	San Marcos	3, 4	
20175		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20176		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20177		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20178		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20179		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20180		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20181		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20182		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20183		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20184		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20185		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20186		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20187		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20188		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20189		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20190		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20191		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20192		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20193		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20194		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20195		male	04/21/2006	30.20932	-97.24291	USA	Texas	Bastrop	BAN04p	GLR pond 5	3, 4	
20198		female	04/22/2006	30.31797	-97.12165	USA	Texas	Lee	LEE14s	Lee Co 333	3, 4	
20199	nebD	female	04/23/2006	30.2875	-97.2312	USA	Texas	Bastrop	BAN39s	Marlin Rd	3, 4	
20342		female	02/22/2006	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
20343		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20344		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20345		female	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
20346		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20347		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20348		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20349		female	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20350		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20351		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20352		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20353		female	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20354		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20355		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20356		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20357		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20358		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20359		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20360		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20361		female	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20362		male	04/22/2006	30.19489	-97.24358	USA	Texas	Bastrop	BAPp	GLR pond 12	3, 4	
20742		female	06/18/2006	29.90066	-96.57999	USA	Texas	Colorado	COL19s	On FM-1291, just E of Lone Oak	3, 4	
20743		female	06/18/2006	29.87021	-96.559	USA	Texas	Colorado	COL04s	At jct of FM-1291 & Ehlinger Rd	3, 4	
20744	nebA	male	06/18/2006	29.87067	-96.55261	USA	Texas	Colorado	COL20s	On FM-1291, on W edge of Frelsburg	3, 4	
20745		female	06/18/2006	29.85117	-96.54633	USA	Texas	Colorado	COL03s	At jct of FM-109 & Dungen Mills Rd	3, 4	
20746		male	06/18/2006	29.85117	-96.54633	USA	Texas	Colorado	COL03s	At jct of FM-109 & Dungen Mills Rd	3, 4	
20747		female	06/18/2006	29.82978	-96.541	USA	Texas	Colorado	COL02s	At jct of FM-109 & CR-42	3, 4	
20748		female	06/18/2006	29.82895	-96.53561	USA	Texas	Colorado	COL17s	On CR-42, 0.5 mi E jct of FM-109 & CR-42	3, 4	
20749		male	06/18/2006	29.82895	-96.53561	USA	Texas	Colorado	COL17s	On CR-42, 0.5 mi E jct of FM-109 & CR-42	3, 4	
20750		female	06/18/2006	29.83018	-96.52185	USA	Texas	Colorado	COL13s	On CR-42	3, 4	
20751		female	06/18/2006	29.83018	-96.52185	USA	Texas	Colorado	COL13s	On CR-42	3, 4	
20752		male	06/18/2006	29.8295	-96.52106	USA	Texas	Colorado	COL18s	On CR-42, near mailbox "1749"	3, 4	
20753		female	06/18/2006	29.82626	-96.51799	USA	Texas	Colorado	COL14s	On CR-42	3, 4	
20754		male	06/18/2006	29.82626	-96.51799	USA	Texas	Colorado	COL14s	On CR-42	3, 4	
20755		female	06/18/2006	29.82458	-96.51604	USA	Texas	Colorado	COL15s	On CR-42	3, 4	
20756		male	06/18/2006	29.82458	-96.51604	USA	Texas	Colorado	COL15s	On CR-42	3, 4	
20757		male	06/18/2006	29.82352	-96.5151	USA	Texas	Colorado	COL16s	On CR-42	3, 4	
20758		female	06/18/2006	29.74697	-96.44168	USA	Texas	Colorado	COL21s	On Mentz Rd	3, 4	
20759		female	06/18/2006	29.74102	-96.44693	USA	Texas	Colorado	COL22s	On Mentz Rd	3, 4	
20760		female	06/18/2006	29.74102	-96.44693	USA	Texas	Colorado	COL22s	On Mentz Rd	3, 4	
20761		male	06/18/2006	29.73178	-96.45678	USA	Texas	Colorado	COL23s	On Mentz Rd	3, 4	
20762		male	06/18/2006	29.71866	-96.47491	USA	Texas	Colorado	COL24s	On Mentz Rd	3, 4	
20764		male	06/18/2006	30.58793	-96.57816	USA	Texas	Burleson	BUR11s	CR-224	3, 4	
20765		female	06/18/2006	30.59821	-96.56111	USA	Texas	Burleson	BUR05s	Jct of CR-229 & CR-224	3, 4	
20766		male	06/18/2006	30.59762	-96.62485	USA	Texas	Burleson	BUR04s	0.5 mi from jct of TX Hwy 21 & FM-1362, on 1362	3, 4	
20767		male	06/18/2006	30.6099	-96.65283	USA	Texas	Burleson	BUR10s	CR-205	3, 4	
20768		female	06/18/2006	30.60906	-96.6527	USA	Texas	Burleson	BUR06s	CR-205	3, 4	
20769		male	06/18/2006	30.60721	-96.6522	USA	Texas	Burleson	BUR07s	CR-205	3, 4	
20770		male	06/18/2006	30.59981	-96.65269	USA	Texas	Burleson	BUR08s	CR-205	3, 4	
20771		female	06/18/2006	30.5987	-96.65307	USA	Texas	Burleson	BUR09s	CR-205	3, 4	
20772		male	06/18/2006	30.57505	-96.66113	USA	Texas	Burleson	BUR03s	0.5 mi from jct of TX Hwy 21 & CR-205, on 205	3, 4	
20773		male	06/18/2006	30.57303	-96.65971	USA	Texas	Burleson	BUR02s	0.5 mi from jct of TX Hwy 21 & CR-205, on 205	3, 4	
20774	nebA	female	06/18/2006	30.41526	-96.64544	USA	Texas	Burleson	BUR01s	0.5 mi from jct of FM-976 & CR-150, on 150	3, 4	
20775	nebC	female	06/18/2006	30.41526	-96.64544	USA	Texas	Burleson	BUR01s	0.5 mi from jct of FM-976 & CR-150, on 150	3, 4	
20776		male	06/18/2006	31.37573	-95.82808	USA	Texas	Leon	LEO03s	FM-3178 near North Creek Baptist Church	3, 4	
20777	nebA	male	06/18/2006	31.37573	-95.82808	USA	Texas	Leon	LEO03s	FM-3178 near North Creek Baptist Church	3, 4	
20778		male	06/18/2006	31.37573	-95.82808	USA	Texas	Leon	LEO03s	FM-3178 near North Creek Baptist Church	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
20779		male	06/18/2006	31.37573	-95.82808	USA	Texas	Leon	LEO03s	FM-3178 near North Creek Baptist Church	3, 4	
20780		male	06/18/2006	31.37573	-95.82808	USA	Texas	Leon	LEO03s	FM-3178 near North Creek Baptist Church	3, 4	
20781		male	06/18/2006	31.37573	-95.82808	USA	Texas	Leon	LEO03s	FM-3178 near North Creek Baptist Church	3, 4	
20782	nebA	male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20783		male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20784		male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20785		male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20786		female	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20787		male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20788		male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20789		male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20790		male	06/18/2006	31.38305	-95.80617	USA	Texas	Leon	LEO02s	FM-3178	3, 4	
20791		female	06/18/2006	30.39996	-96.64024	USA	Texas	Burleson	BUR12s	CR-408	3, 4	
20792		male	06/18/2006	30.39568	-96.65129	USA	Texas	Burleson	BUR14s	FM-2774	3, 4	
20793		female	06/18/2006	29.4469	-97.1794	USA	Texas	Lavaca	LAV01s	cemetery by Green Dickson Park	3, 4	
20794		male	06/18/2006	29.44631	-97.17803	USA	Texas	Lavaca	LAV10s	Shiner	3, 4	
20796		female	06/18/2006	30.60852	-96.65264	USA	Texas	Burleson	BUR16s	CR-205	4	
20797		female	06/18/2006	29.45069	-97.18173	USA	Texas	Lavaca	LAV08s	near Green Dickson Park, Shiner	3, 4	
20798		female	06/18/2006	29.44631	-97.17883	USA	Texas	Lavaca	LAV04s	CR-348	3, 4	
20799		male	06/18/2006	29.44631	-97.17883	USA	Texas	Lavaca	LAV04s	CR-348	3, 4	
20800		female	06/18/2006	29.43923	-97.16923	USA	Texas	Lavaca	LAV05s	FM-3435	3, 4	
20801		male	06/18/2006	29.43923	-97.16923	USA	Texas	Lavaca	LAV05s	FM-3435	3, 4	
20802		male	06/18/2006	29.43923	-97.16923	USA	Texas	Lavaca	LAV05s	FM-3435	3, 4	
20803	nebC	male	06/18/2006	29.45069	-97.18173	USA	Texas	Lavaca	LAV08s	near Green Dickson Park, Shiner	3, 4	
20804		female	06/18/2006	29.45069	-97.18173	USA	Texas	Lavaca	LAV08s	near Green Dickson Park, Shiner	3, 4	
20805		male	06/18/2006	29.45069	-97.18173	USA	Texas	Lavaca	LAV08s	near Green Dickson Park, Shiner	3, 4	
20806		male	06/18/2006	29.45069	-97.18173	USA	Texas	Lavaca	LAV08s	near Green Dickson Park, Shiner	3, 4	
20807		male	06/18/2006	29.43923	-97.17131	USA	Texas	Lavaca	LAV07s	FM-3435, 1 mi N of jct w/ 90A	3, 4	
20808		female	06/18/2006	29.4469	-97.17794	USA	Texas	Lavaca	LAV09s	near cemetery	3, 4	
20809		female	06/18/2006	29.4469	-97.17794	USA	Texas	Lavaca	LAV09s	near cemetery	3, 4	
20810		female	06/18/2006	29.43913	-97.17037	USA	Texas	Lavaca	LAV06s	FM-3435	3, 4	
20811		male	06/18/2006	29.43913	-97.17037	USA	Texas	Lavaca	LAV06s	FM-3435	3, 4	
20824		male	06/20/2006	30.54236	-96.7025	USA	Texas	Burleson	BUR15s	TX Hwy 36 at N city limit of Caldwell	3, 4	
20828	nebA	male	06/18/2006	29.73291	-96.42121	USA	Texas	Colorado	COL08s	FM-949	3, 4	
20829		female	06/18/2006	29.73247	-96.42213	USA	Texas	Colorado	COL09s	FM-949	3, 4	
20831		female	06/18/2006	29.73353	-96.41985	USA	Texas	Colorado	COL10s	FM-949	3, 4	
20833	nebA	female	06/18/2006	29.88982	-96.30812	USA	Texas	Austin	AUS04s	Mill Creek Rd	3, 4	
20834		male	06/18/2006	29.87681	-96.31247	USA	Texas	Austin	AUS08s	Mill Creek Rd	3, 4	
20836		female	06/18/2006	29.89713	-96.30732	USA	Texas	Austin	AUS05s	Mill Creek Rd	3, 4	
20837		female	06/18/2006	29.73684	-96.41606	USA	Texas	Colorado	COL11s	FM-949	3, 4	
20838		female	06/18/2006	29.72921	-96.42245	USA	Texas	Colorado	COL12s	FM-949, 0.5 mi N of jct w/ I-10	3, 4	
20840		female	06/18/2006	29.87404	-96.3131	USA	Texas	Austin	AUS06s	Mill Creek Rd	3, 4	
20841		male	06/18/2006	29.87515	-96.31257	USA	Texas	Austin	AUS13s	Mill Creek Rd, 0.25 mi from jct 2187	3, 4	
20842		male	06/18/2006	29.87649	-96.31267	USA	Texas	Austin	AUS07s	Mill Creek Rd	3, 4	
20843		male	06/18/2006	29.87649	-96.31267	USA	Texas	Austin	AUS07s	Mill Creek Rd	3, 4	
20844		male	06/18/2006	29.87649	-96.31267	USA	Texas	Austin	AUS07s	Mill Creek Rd	3, 4	
20845		female	06/18/2006	29.87649	-96.31267	USA	Texas	Austin	AUS07s	Mill Creek Rd	3, 4	
20846		male	06/18/2006	29.87649	-96.31267	USA	Texas	Austin	AUS07s	Mill Creek Rd	3, 4	
20847		female	06/18/2006	29.87649	-96.31267	USA	Texas	Austin	AUS07s	Mill Creek Rd	3, 4	
20848		male	06/18/2006	29.87681	-96.31247	USA	Texas	Austin	AUS08s	Mill Creek Rd	3, 4	
20849		male	06/18/2006	29.87718	-96.31211	USA	Texas	Austin	AUS09s	Mill Creek Rd	3, 4	
20850		female	06/18/2006	29.87718	-96.31211	USA	Texas	Austin	AUS09s	Mill Creek Rd	3, 4	
20851		male	06/18/2006	29.91759	-96.30168	USA	Texas	Austin	AUS10s	Mill Creek Rd	3, 4	
20852		female	06/18/2006	29.91759	-96.30168	USA	Texas	Austin	AUS10s	Mill Creek Rd	3, 4	
20853		male	06/18/2006	29.91759	-96.30168	USA	Texas	Austin	AUS10s	Mill Creek Rd	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
20855	MF20855	female	06/18/2006	30.78961	-96.27118	USA	Texas	Brazos	BRA04s	FM-2038, 4.9 mi SE jct 974/2038	3, 4	HM021127
20856		male	06/18/2006	30.78961	-96.27118	USA	Texas	Brazos	BRA04s	FM-2038, 4.9 mi SE jct 974/2038	3, 4	
20857		male	06/18/2006	30.78961	-96.27118	USA	Texas	Brazos	BRA04s	FM-2038, 4.9 mi SE jct 974/2038	3, 4	
20858		male	06/18/2006	30.78961	-96.27118	USA	Texas	Brazos	BRA04s	FM-2038, 4.9 mi SE jct 974/2038	3, 4	
20859		male	06/18/2006	30.78961	-96.27118	USA	Texas	Brazos	BRA04s	FM-2038, 4.9 mi SE jct 974/2038	3, 4	
20860		female	06/20/2006	30.6505	-96.46706	USA	Texas	Brazos	BRA05s	NE of by-pass & S of OSR	3, 4	
20861		female	06/20/2006	30.6505	-96.46706	USA	Texas	Brazos	BRA05s	NE of by-pass & S of OSR	3, 4	
20862		female	06/20/2006	30.6505	-96.46706	USA	Texas	Brazos	BRA05s	NE of by-pass & S of OSR	3, 4	
20863		female	06/20/2006	30.6505	-96.46706	USA	Texas	Brazos	BRA05s	NE of by-pass & S of OSR	3, 4	
20864		female	06/20/2006	30.6505	-96.46706	USA	Texas	Brazos	BRA05s	NE of by-pass & S of OSR	3, 4	
20865		female	06/20/2006	30.6505	-96.46706	USA	Texas	Brazos	BRA05s	NE of by-pass & S of OSR	3, 4	
20866	nebD	male	06/20/2006	30.85636	-96.53686	USA	Texas	Robertson	ROB01s	FM-2599, 2.8 mi S jct 391/2599	3, 4	
20867	nebA	male	06/20/2006	30.89853	-96.43569	USA	Texas	Robertson	ROB02s	FM-391, 3.1 mi W of jct w/ FM-46	3, 4	
20943	nebA	female	06/23/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
20944	nebA	female	06/23/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
20960	MF20960	male	06/23/2006	28.2403	-96.8344	USA	Texas	Aransas	ARA01s	Aransas National Wildlife Refuge Loop Trail	3, 4	HM021128
20961	nebA	male	06/23/2006	28.2403	-96.8344	USA	Texas	Aransas	ARA01s	Aransas National Wildlife Refuge Loop Trail	3, 4	
20962	nebA	male	06/23/2006	28.2403	-96.8344	USA	Texas	Aransas	ARA01s	Aransas National Wildlife Refuge Loop Trail	3, 4	
20963	nebA	female	06/23/2006	28.2403	-96.8344	USA	Texas	Aransas	ARA01s	Aransas National Wildlife Refuge Loop Trail	3, 4	
21488		male	08/24/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
21498	nebC	female	05/02/2006	30.18083	-96.59769	USA	Texas	Washington	WAS01s	0.1mi ENE Indian Creek Crossing on FM390	3, 4	
21559		unknown	06/19/2006	29.47257	-95.6458	USA	Texas	Ft. Bend	FTB05s	Smithers & Rabbs	3, 4	
21560	nebA	unknown	06/19/2006	29.47257	-95.6458	USA	Texas	Ft. Bend	FTB05s	Smithers & Rabbs	3, 4	
21561		male	06/19/2006	29.47257	-95.6458	USA	Texas	Ft. Bend	FTB05s	Smithers & Rabbs	3, 4	
21562		unknown	06/19/2006	29.41467	-95.6421	USA	Texas	Ft. Bend	FTB02s	Oak Dr & Rawlings	3, 4	
21563		male	06/19/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21564		male	06/19/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21565		male	06/19/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21566	nebA	male	06/19/2006	29.39735	-95.62511	USA	Texas	Ft. Bend	FTB01s	5710 Sawmill	3, 4	
21567		female	06/19/2006	29.39735	-95.62511	USA	Texas	Ft. Bend	FTB01s	5710 Sawmill	3, 4	
21568	nebA	male	06/21/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	3, 4	
21569		female	06/21/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	3, 4	
21570		female	06/21/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	3, 4	
21571		male	06/21/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	3, 4	
21572		male	06/21/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	3, 4	
21573		female	06/21/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	3, 4	
21574		unknown	06/21/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	3, 4	
21575	nebB	male	06/18/2006	30.033	-94.90448	USA	Texas	Liberty	LIB01s	CR-493	3, 4	
21576	nebA	male	06/18/2006	30.03193	-94.9045	USA	Texas	Liberty	LIB02s	CR-493	3, 4	
21577		unknown	06/18/2006	30.03193	-94.9045	USA	Texas	Liberty	LIB02s	CR-493	3, 4	
21578		unknown	06/18/2006	30.03193	-94.9045	USA	Texas	Liberty	LIB02s	CR-493	3, 4	
21579		female	06/18/2006	30.02978	-94.90448	USA	Texas	Liberty	LIB03s	CR-493	3, 4	
21580		female	06/18/2006	30.02867	-94.90448	USA	Texas	Liberty	LIB04s	CR-493	3, 4	
21581		unknown	06/18/2006	30.02055	-94.90446	USA	Texas	Liberty	LIB05s	CR-493	3, 4	
21582		female	06/18/2006	30.01875	-94.90446	USA	Texas	Liberty	LIB06s	CR-493	3, 4	
21583		female	06/18/2006	30.01875	-94.90446	USA	Texas	Liberty	LIB06s	CR-493	3, 4	
21584		unknown	06/18/2006	30.01875	-94.90446	USA	Texas	Liberty	LIB06s	CR-493	3, 4	
21586		unknown	06/18/2006	30.0257	-94.90442	USA	Texas	Liberty	LIB07s	CR-493	3, 4	
21587		female	06/18/2006	30.02695	-94.90448	USA	Texas	Liberty	LIB08s	CR-493	3, 4	
21588		female	06/18/2006	30.02867	-94.9045	USA	Texas	Liberty	LIB09s	CR-493	3, 4	
21589		female	06/18/2006	30.0306	-94.9045	USA	Texas	Liberty	LIB10s	CR-493	3, 4	
21590		unknown	06/18/2006	30.03165	-94.90446	USA	Texas	Liberty	LIB11s	CR-493	3, 4	
21591		female	06/18/2006	30.03248	-94.90443	USA	Texas	Liberty	LIB12s	CR-493	3, 4	
21592		unknown	06/18/2006	30.03248	-94.90443	USA	Texas	Liberty	LIB12s	CR-493	3, 4	
21593		male	06/18/2006	30.03248	-94.90443	USA	Texas	Liberty	LIB12s	CR-493	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
21594		unknown	06/18/2006	30.03043	-94.90448	USA	Texas	Liberty	LIB13s	CR-493	3, 4	
21602	nebB	juvenile	09/16/2006	23.17525	-98.43585	Mexico	Tamaulipas		MEX02s	3.5 mi SE Acuna	3, 4	
21603		male	09/16/2006	23.20467	-98.43681	Mexico	Tamaulipas		MEX09s	Rancho Acuna	3, 4	
21629		female	09/18/2006	23.21861	-98.4575	Mexico	Tamaulipas		MEX07s	N of La Vibora on road	3, 4	
21633	nebA	male	09/18/2006	23.03964	-98.83225	Mexico	Tamaulipas		MEX01s	10 mi E of Xicocentath	3, 4	HM021129
21634	nebA	male	09/18/2006	23.03964	-98.83225	Mexico	Tamaulipas		MEX01s	10 mi E of Xicocentath	3, 4	
21635		male	09/18/2006	23.03964	-98.83225	Mexico	Tamaulipas		MEX01s	10 mi E of Xicocentath	3, 4	
21636		male	09/18/2006	23.03964	-98.83225	Mexico	Tamaulipas		MEX01s	10 mi E of Xicocentath	3, 4	
21681			09/23/2006	22.91333	-99.48833	Mexico	Tamaulipas		MEX10s	Santa Maria de Guadalupe Ejido along Rio Campo	3, 4	
21682			09/23/2006	22.91333	-99.48833	Mexico	Tamaulipas		MEX10s	Santa Maria de Guadalupe Ejido along Rio Campo	3, 4	
21705		female	09/25/2006	22.83444	-99.57359	Mexico	Tamaulipas		MEX11s	Tula rd to Lagunas Escondidas	3, 4	
21860		female	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21861	nebA	female	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21862		male	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21864		male	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21865		unknown	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21866		male	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21867		male	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21868		unknown	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21869		male	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21871		male	06/19/2006	29.71265	-95.3918	USA	Texas	Harris	HAR02s	Houston Zoo	3, 4	
21872		male	05/06/2006	29.8816	-96.30952	USA	Texas	Austin	AUS11s	Mill Creek Rd	3, 4	
21873		male	05/06/2006	29.89038	-96.30805	USA	Texas	Austin	AUS12s	Mill Creek Rd	3, 4	
21874		female	05/06/2006	29.89038	-96.30805	USA	Texas	Austin	AUS12s	Mill Creek Rd	3, 4	
21875		female	05/06/2006	29.89038	-96.30805	USA	Texas	Austin	AUS12s	Mill Creek Rd	3, 4	
21876		male	06/21/2006	29.45962	-95.64602	USA	Texas	Ft. Bend	FTB03s	Rabbs Rd	3, 4	
21877		male	06/21/2006	29.45962	-95.64602	USA	Texas	Ft. Bend	FTB03s	Rabbs Rd	3, 4	
21878		male	06/21/2006	29.45962	-95.64602	USA	Texas	Ft. Bend	FTB03s	Rabbs Rd	3, 4	
21879		male	06/21/2006	29.45865	-95.646	USA	Texas	Ft. Bend	FTB04s	Rabbs Rd	3, 4	
21880		male	06/21/2006	29.45865	-95.646	USA	Texas	Ft. Bend	FTB04s	Rabbs Rd	3, 4	
21881		male	06/21/2006	29.47257	-95.6458	USA	Texas	Ft. Bend	FTB05s	Smithers & Rabbs	3, 4	
21882		unknown	06/21/2006	29.47257	-95.6458	USA	Texas	Ft. Bend	FTB05s	Smithers & Rabbs	3, 4	
21883		male	06/21/2006	29.47257	-95.6458	USA	Texas	Ft. Bend	FTB05s	Smithers & Rabbs	3, 4	
21884		male	06/21/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21885		male	06/21/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21886		male	06/21/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21887		male	06/21/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21888		male	06/21/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21889		male	06/21/2006	29.40465	-95.6529	USA	Texas	Ft. Bend	FTB06s	W Cumings & 762	3, 4	
21890		male	06/22/2006	29.68737	-95.50301	USA	Texas	Harris	HAR01s	8506 Bob White Dr	4	
22062		male	08/07/2002			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	
22063	nebE		08/07/2002			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	
22064			08/07/2002			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	
22083			08/07/2002			USA	Texas	Cameron	CAM01p	Southmost Preserve	3, 4	
22162	MF22162	female	10/13/2006	23.61883	-99.258	Mexico	Tamaulipas		MEX08s	On HWY 101 near La Reforma, 13.5 km SW of Victoria	3, 4	HM021130
22283		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	3, 4	
22358		male	03/14/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	
22375	nebA	female	03/12/2007	29.34291	-96.83861	USA	Texas	Lavaca	LAV02s	CR-15	3, 4	
22376		female	03/15/2007	29.36116	-96.82395	USA	Texas	Lavaca	LAV03s	CR-16a, about 1.5 km NW of jct w/ CR-16	3, 4	
22377		female	03/15/2007	29.36116	-96.82395	USA	Texas	Lavaca	LAV03s	CR-16a, about 1.5 km NW of jct w/ CR-16	3, 4	
22378		male	03/15/2007	29.36116	-96.82395	USA	Texas	Lavaca	LAV03s	CR-16a, about 1.5 km NW of jct w/ CR-16	3, 4	
22382		female	02/12/2007	29.83003	-96.48025	USA	Texas	Colorado	COL50s	CR-52	3, 4	
22383		female	02/28/2007	29.84215	-96.49125	USA	Texas	Colorado	COL06s	CR-52	3, 4	
22384		male	03/20/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
22385		male	03/20/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
22386		male	03/20/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
22387		male	03/20/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
22388		male	03/20/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
22391		male	03/25/2007	30.13288	-97.26572	USA	Texas	Bastrop	BAS01p	BBHQ pond 1	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
22392		male	03/25/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
22393		male	03/25/2007	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	3, 4	
22399	nebA	male	03/27/2007	30.72427	-96.84543	USA	Texas	Milam	MIL02s	CR-339	3, 4	
22400	nebA	male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22401		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22402		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22403		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22404		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22405		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22406		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22407		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22408		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22409		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22410		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22411		female	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22412		male	03/27/2007	30.73318	-96.67779	USA	Texas	Milam	MIL05p	Cernuch pond	3, 4	
22413		male	03/27/2007	30.71423	-96.85377	USA	Texas	Milam	MIL03s	CR-339 at jct w/ 190/79	3, 4	
22414		female	03/27/2007	30.71423	-96.85377	USA	Texas	Milam	MIL03s	CR-339 at jct w/ 190/79	3, 4	
22415		male	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	3, 4	
22416		male	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	3, 4	
22417		female	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	3, 4	
22421		male	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	3, 4	
22422		male	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	3, 4	
22426	nebA	male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22427	nebA	male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22428		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22429		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22430		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22431		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22432		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22433		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22434		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22435		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22436		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22437		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22438		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22439		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22440		male	03/27/2007	30.13941	-97.25118	USA	Texas	Bastrop	BAS14p	Jim Small pond 1	3, 4	
22458	nebE	male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22459	nebG	male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22460		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22461		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22462		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22463		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22464		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22465		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22466		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22467		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22468		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22469		male	03/10/2007	25.85325	-97.39642	USA	Texas	Cameron	CAM01p	Southmost Preserve Resaca Unit #1	3, 4	
22473		female	03/27/2007	30.17795	-97.2338	USA	Texas	Bastrop	BAN11p	GLR pond 15	3, 4	
22474		male	03/27/2007	30.17795	-97.2338	USA	Texas	Bastrop	BAN11p	GLR pond 15	3, 4	
22475		male	03/27/2007	30.17873	-97.23247	USA	Texas	Bastrop	BAN38p	GLR pond 14	3, 4	
22476		male	03/27/2007	30.17873	-97.23247	USA	Texas	Bastrop	BAN38p	GLR pond 14	3, 4, 5	HM021065
22477		male	03/27/2007	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
22478		male	03/27/2007	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	
22479		male	03/27/2007	30.1978	-97.21326	USA	Texas	Bastrop	BAN09p	GLR pond 10	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo nebulifer* continued**												
22480	nebA	male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22481	nebA	male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22482		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22484		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22485		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22486		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22487		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22488		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22489		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22490		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22491		female	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22492		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22493		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22494		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22495		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22496		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22497		male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	3, 4	
22527		male	03/27/2007	30.71423	-96.85377	USA	Texas	Milam	MIL03s	CR-339 at jct w/ 190/79	3, 4	
22528		male	04/03/2007	30.75997	-96.68343	USA	Texas	Milam	MIL04s	CR-358, 0.5 km E of CR-359	3, 4	
22721		female	06/24/2007	31.03957	-96.5956	USA	Texas	Robertson	ROB03s	FM-979, 7 km WSW of jct w/ FM-46	3, 4	
22724	nebA	male	??/??/2007	29.8417	-96.48763	USA	Texas	Colorado	COL07s	CR-52, 75 m E of jct w/ Warsehak Schuette Rd	3, 4	
22725	nebA	female	??/??/2007	29.8417	-96.48763	USA	Texas	Colorado	COL07s	CR-52, 75 m E of jct w/ Warsehak Schuette Rd	3, 4	
22726	nebB		??/??/2007	30.39706	-96.23872	USA	Texas	Brazos	BRA03s	FM-159, 6.3 rd mi S of FM-2154	3, 4	
22727		unknown	05/26/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, pond in backyard	3, 4	
22728		unknown	05/26/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, pond in backyard	3, 4	
22729		unknown	05/26/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, pond in backyard	3, 4	
22730		unknown	05/26/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, pond in backyard	3, 4	
22731		unknown	05/26/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, pond in backyard	3, 4	
22732		unknown	05/26/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, pond in backyard	3, 4	
22733		unknown	05/26/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, pond in backyard	3, 4	
22752	nebA	juvenile	07/08/2007	29.96742	-97.31706	USA	Texas	Bastrop	BAS20s	Davis property, S of Colorado R	3, 4	
23141			03/15/2007			USA	Texas	Aransas	ARA01s	Aransas National Wildlife Refuge	3, 4	
23198		juvenile	05/16/2007	30.63443	-96.34154	USA	Texas	Brazos	BRA02s	Bryan, 705 Inwood, in yard	3, 4	
23405		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23406		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23407		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23408		female	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23409		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23410		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23411		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23412		juvenile	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23413		juvenile	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23414		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23415		female	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	
23416		juvenile	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	3, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
Bufo woodhousii												
3523	wooC	juvenile	06/03/2000			USA	Texas	Wichita	WIC01s	unknown rd w/in 1 mi radius of Burkburnett	2, 4, 5	HM021032, HM021036, HM021053, HM021080
5270	wooB	male	09/10/2001	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	HM021101
5271	wooB		09/10/2001	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
5272	wooA		09/10/2001	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	HM021040, HM021102
5273	wooB		09/10/2001	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
5274	wooA		09/10/2001	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	HM021059
7398	MF07398	male	04/17/2002			USA	Oklahoma	Cleavand	OKL01s	Sutton Wilderness Area in Norman	2, 4	HM021104
10031	wooA	female	04/29/2004	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
20085	wooB	female	04/10/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	HM021070, HM021074, HM021078
20086	wooB	female	04/10/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
20087	wooB	female	04/10/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	HM021052
20088	wooA	female	04/10/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
20089	wooB	male	04/10/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
20945	wooB	male	06/23/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
20946	wooA	male	06/23/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
20947	wooB	male	06/23/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	HM021087
20948	wooC	female	06/25/2006			USA	Oklahoma	Potowatomi	OKL02s	Shawnee, Market St, in yard	2, 4, 5	HM021047, HM021114
21487	wooB	male	08/24/2006	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	2, 4, 5	
22054	wooE	female	07/01/2002	28.11433	-96.80694	USA	Texas	Aransas	ARA02s	Padre Island, Corpus Christi Beach	2, 4	HM021115
22055	wooE	male	07/15/2002	28.11433	-96.80694	USA	Texas	Aransas	ARA02s	Padre Island, Corpus Christi Beach	2, 4	
23417		female	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	4, 5	
23418		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	4, 5	
23419		juvenile	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	4, 5	
23428		male	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	4, 5	
23429		juvenile	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	4, 5	
23430		juvenile	07/28/2007	32.10653	-97.31817	USA	Texas	Hill	HIL01s	FM-934, 4 mi E of jct w/ FM-933	4, 5	
Putative hybrids												
3651	wooC	male	02/24/2001	30.20997	-97.24821	USA	Texas	Bastrop	BAN32p	GLR pond 3	4	
3802	wooA	male	04/04/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
16663		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
20057	houC	male	03/30/2006	30.10839	-97.25984	USA	Texas	Bastrop	BAS19p	BSP pond 9	4	
20059	houB	male	03/30/2006	30.09016	-97.23851	USA	Texas	Bastrop	BAS09p	BSP pond 19	4	
22369	houC	male	03/14/2007	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	4	
22419	nebC	male	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	4	
22420	houB	male	03/27/2007	30.12633	-97.2337	USA	Texas	Bastrop	BAS18p	Jim Small pond 5	4	
22498	nebD	male	03/29/2007	30.24567	-97.22135	USA	Texas	Bastrop	BAN28p	Musgrave Pond	4	
Predefined hybrids												
3619	wooA	Egg	02/24/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3620	wooA	Egg	02/24/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3621	wooA	Egg	02/24/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3622	wooA	Egg	02/24/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
3631	wooC	male	02/23/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
3650	nebB	adult	02/20/2001	30.21427	-97.23254	USA	Texas	Bastrop	BAN05p	GLR pond 6	4	
3803	houA	male	04/04/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
3901	wooA	tadpole	04/06/2001	30.32482	-97.16896	USA	Texas	Lee	LEE02p	Durham pond 1	2, 4	

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
Predefined hybrids continued												
4445	wooC	tadpole	04/16/2001	30.36224	-97.25602	USA	Texas	Bastrop	BAN31p	E3 pond 6	4	
4867	wooA	juvenile	04/17/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
4869	wooC	juvenile	05/26/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
4871	nebA	juvenile	06/09/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
4873	nebA	juvenile	06/11/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
4874	nebA	juvenile	06/11/2001	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
5718	wooA	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5719	wooA	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5721	wooA	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5722	wooA	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5723	wooA	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
5724	wooA	tadpole	04/17/2002	30.32764	-97.16957	USA	Texas	Lee	LEE03p	Durham pond 2	2, 4	
8911		male	04/08/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	4	
8997	wooA	juvenile	05/28/2003			USA	Texas	Bastrop	BAN04p	GLR B-F4	2, 4	
9070	wooA	male	03/07/2002	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
9349	wooA		03/10/2003	30.21647	-97.24178	USA	Texas	Bastrop	BAN13t	GLR 2-1	2, 4	
16653	wooA		02/18/2002			USA	Texas	Bastrop	BAN02p	GLR 1-N	2, 4	
16654	wooA		02/18/2002			USA	Texas	Bastrop	BAN02p	GLR 1-N	2, 4	
16716	wooA	male	02/13/2003	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1-1	2, 4	
16990	nebA	tadpole	05/08/2004	30.13721	-97.24335	USA	Texas	Bastrop	BAS15p	Jim Small pond 2	4	
17099	houA	male	03/11/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	4	
17100		male	03/11/2003	30.14236	-97.1958	USA	Texas	Bastrop	BAS06p	Bob Long Back Pond	4	
20010		male	03/30/2006	30.12638	-97.23934	USA	Texas	Bastrop	BAS17p	Jim Small pond 4	4	
21332	nebA	juvenile	06/14/2003	30.21586	-97.23886	USA	Texas	Bastrop	BAN12t	GLR 1-1	4	
22053	nebF	male	03/01/2002	31.0775	-96.19334	USA	Texas	Leon	LEOp	Hilltop Lakes	4	
22121	nebF	male	03/01/2002	31.0775	-96.19334	USA	Texas	Leon	LEOp	Hilltop Lakes	4	
22291		male	02/21/2007	30.11438	-97.27673	USA	Texas	Bastrop	BAS08p	BSP pond 11	4	
22322	wooA	male	02/23/2007	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
22323	wooA	male	02/23/2007	30.21626	-97.24172	USA	Texas	Bastrop	BAN02p	GLR pond 2	2, 4	
22472	wooA	male	03/25/2007	30.23752	-97.21152	USA	Texas	Bastrop	BAN30s	Dube Lane, off US Hwy 290 on dirt road	2, 4	
Bufo americanus												
1103	MF01103		10/01/1996	42.49631	-75.29703	USA	New York	Otsego	n/a	5 mi SW Morris on Rt 51	2, 5	HM021027, HM021048, HM021093
2968	MF02968		09/01/1996	41.38932	-74.0167	USA	New York	Orange	n/a	Black Rock Forest	2	HM021094
7399	MF07399	male	04/16/2002			USA	Oklahoma	Cleavand	OKL01s	Sutton Wilderness Area in Norman	2	HM021105
8153	wooD	male	05/01/2003	36.57712	-93.29542	USA	Missouri	Taney	n/a	Jct Dale Rd & Hwy 165	2, 5	HM021061, HM021091, HM021107
8154	MF08154	male	05/01/2003	36.57712	-93.29542	USA	Missouri	Taney	n/a	Jct Dale Rd & Hwy 165	2, 5	HM021038, HM021085, HM021108
8155	wooD	male	05/01/2003	36.57712	-93.29542	USA	Missouri	Taney	n/a	Jct Dale Rd & Hwy 165	2, 5	HM021081
8156	wooD	male	05/01/2003	36.57712	-93.29542	USA	Missouri	Taney	n/a	Jct Dale Rd & Hwy 165	2, 5	HM021034
8157	wooD	male	05/01/2003	36.57712	-93.29542	USA	Missouri	Taney	n/a	Jct Dale Rd & Hwy 165	2, 5	HM021041, HM021044
Bufo baxteri												
20450			01/28/2005			USA	Wyoming		n/a	From Wyoming via Houston Zoo	5	HM021064
Bufo coccifer												
4059		female	05/29/1999	9.79881	-84.58683	Costa Rica	Puntarenas		n/a	Reserva Biologica Carara 1.9 rd mi S of Tarcoles River on Hwy 34	5	
Bufo cognatus												
3525	MF03525	male	06/03/2000			USA	Texas	Wichita	WIC01s	unknown rd w/in 1 mi radius of Burkburnett	2, 5	HM021037, HM021095
27040	cogA	juvenile	09/05/2008	34.90861	-102.1189	USA	Texas	Randall	n/a	Buffalo Lakes WR at campground	2	
27054	cogA		09/05/2008	34.62028	-102.6047	USA	Texas	Parmer	n/a	7.6 mi N jct w/ TX86	2	HM021118
Bufo debilis												
21446		female	08/19/2006	30.58806	-104.6333	USA	Texas	Jeff Davis	n/a	Miller Ranch, Kimball house dump	5	
Bufo fowleri												
5186	MF05186		06/10/2001	33.64994	-85.05634	USA	Georgia	Carroll	n/a	Carrollton, 5 mi NE of jct 27 &113, lake N of 113	2, 5	HM021050, HM021058, HM021100

MF no.	mtDNA haplotype	Sex	Date sampled	Latitude	Longitude	Country	State	County	Site code	Locality description	Chapter(s)	GenBank Accession(s)
***Bufo fowleri* continued**												
10100	fowA	female	07/27/2004	38.30851	-77.37448	USA	Virginia	Stafford	n/a	0.6 mi N jct of RD 218 & 203 on 203	2, 5	HM021026, HM021110
10102		male	07/27/2004	38.30851	-77.37448	USA	Virginia	Stafford	n/a	0.6 mi N jct of RD 218 & 203 on 203	5	HM021045
10103	fowA	female	07/27/2004	38.30851	-77.37448	USA	Virginia	Stafford	n/a	0.6 mi N jct of RD 218 & 203 on 203	2, 5	HM021042, HM021069, HM021073, HM021077, HM021086
10108			07/27/2004	38.30851	-77.37448	USA	Virginia	Stafford	n/a	0.6 mi N jct of RD 218 & 203 on 203	5	HM021029
10109			07/27/2004	38.30851	-77.37448	USA	Virginia	Stafford	n/a	0.6 mi N jct of RD 218 & 203 on 203	5	
Bufo marinus												
4009		female	06/09/1999	9.79411	-84.59764	Costa Rica	Puntarenas		n/a	Reserva Biologica Carara, 1.9 rd mi S of Tarcoles River on Hwy 34	5	
4033			06/15/1999			Costa Rica	San Jose		n/a	Reserva Biologica Carara, Montanas Jamaica, 1.4 rd mi N of Bijagual & 0.6 rd mi NNE	5	
4039		female	06/07/1999	9.79881	-84.58683	Costa Rica	Puntarenas		n/a	Reserva Biologica Carara, 1.9 rd mi S of Tarcoles River on Hwy 34	5	
4570			01/07/2001	9.77925	-84.53128	Costa Rica	San Jose		n/a	near Parque Nacional Carara, Bajo Carrara	5	
5360		male	06/22/2001	10.89611	-85.33531	Costa Rica	Alajuela		n/a	Area de Conservacion Guanacaste	5	
5361		male	06/25/2001	10.89611	-85.33531	Costa Rica	Alajuela		n/a	Area de Conservacion Guanacaste	5	
9540		male	07/09/2004	23.41667	-99.33353	Mexico	Tamaulipas		n/a	23.25.019 - 99.20.714	5	
9541		female	07/09/2004	23.40022	-99.35008	Mexico	Tamaulipas		n/a	23.24.828 - 99.21.296	5	HM021024
10260		female	05/31/2003			Costa Rica	Puntarenas		n/a	Reserva Natural Absoluta Cabo Blanco, Puesto Cabuya (Admin HQ)	5	HM021054
16475		female	06/07/2003	9.58058	-85.12461	Costa Rica	Puntarenas		n/a	Reserva Natural Absoluta Cabo Blanco, Sendero Central just S of Laguna Balsitas	5	
20256		male	07/01/2004			Ecuador	Orellana		n/a	Yasuni National Park, TBS, Rio Tiputini, Lago trail meter marker 950	5	HM021063
21596			09/16/2006	23.12306	-98.47037	Mexico	Tamaulipas		n/a	Acuna Rd	5	
21658		juvenile	09/17/2006	23.15333	-98.43567	Mexico	Tamaulipas		n/a	5 km SE Acuna, on road	5	
21661			09/20/2006	22.94358	-98.09186	Mexico	Tamaulipas		n/a	Near Aldama	5	
22101		juvenile	08/08/2002			USA	Texas	Zapata	n/a		5	
22102		juvenile	08/08/2002			USA	Texas	Zapata	n/a		5	
22103		juvenile	08/08/2002			USA	Texas	Zapata	n/a		5	
22107			08/08/2002			USA	Texas	Zapata	n/a		5	
22161		juvenile	10/13/2006	23.61783	-99.27016	Mexico	Tamaulipas		n/a	On HWY 101 near La Reforma, 14 km SW of Victoria	5	
22703			06/09/2007	26.5777	-99.12905	USA	Texas	Starr	n/a	backyard	5	
22704			06/09/2007	26.5777	-99.12905	USA	Texas	Starr	n/a	backyard	5	
22933		male	08/11/2006	-0.63847	-76.14908	Ecuador	Orellana		n/a	Yasuni National Park, volleyball court	5	
22934		male	08/11/2006	-0.63847	-76.14908	Ecuador	Orellana		n/a	Yasuni National Park, volleyball court	5	
Bufo melanochlorus												
4527	MF04527	female	01/31/2000			Costa Rica	San Jose		n/a	Parque Nacional Carara, Montañas Jamaica, 1.4 rd mi N of Bijagual & 0.6 rd mi NNE Quebrada Máquina	3, 5	HM021121
Bufo punctatus												
3672		unknown	06/11/2000	29.62993	-100.42126	USA	Texas	Edwards	EDW01s	Kickapoo Cavern SP	5	HM021084, HM021090
Bufo speciosus												
3524		juvenile	06/03/2000			USA	Texas	Wichita	WIC01s	unknown rd w/in 1 mi radius of Burkburnett	5	HM021049, HM021083, HM021089
Bufo valliceps												
6336	MF06336	juvenile	06/25/2001	10.91392	-85.30169	Costa Rica	Alajuela		n/a	Area de Conservacion Guanacaste, Rio Negro, near Buenos Aires de Aguas Claras de Upala	3, 5	HM021060, HM021122

Appendix B

Voucher specimen records of *Bufo houstonensis*. BSP = Bastrop State Park , GLR = Griffith League Ranch, HZG = Houston Zoological Gardens, and * indicates holotype or paratypes (UIMNH 33687 is holotype). Specimens under Institution TTU are now held at TNHC. Data were obtained from records held in the following institutions and accessed through the HerpNET data portal (http://www.herpnet.org) on 22 July 2009 and the GBIF data portal (http://www.gbif.net) on 23 July 2009: Borror Laboratory of Bioacoustics, Museum of Biological Diversity, Ohio State University, Columbus; California Academy of Sciences, San Francisco; Carnegie Museum, Pittsburgh; Cornell University, Ithaca; Florida Museum of Natural History, University of Florida, Gainesville; Field Museum, Chicago; Illinois Natural History Survey, Champaign; Museum of Natural History, University of Kansas, Lawrence; Natural History Museum of Los Angeles County, Los Angeles; Museum of Natural Science, Louisiana State University, Baton Rouge; Museum of Comparative Zoology, Harvard University, Cambridge; Michigan State University Museum, East Lansing; Museum of Vertebrate Zoology, University of California, Berkeley; San Diego Natural History Museum, San Diego; Texas Cooperative Wildlife Collection, College Station; University of Colorado Museum, Boulder; Museum of Natural History, University of Illinois, Urbana; Museum of Zoology, University of Michigan, Ann Arbor; and National Museum of Natural History, Washington, D.C. Additional data were obtained from records held in the following institutions and provided by David C. Cannatella, Travis J. LaDuc, Toby J. Hibbitts, Trey Crumpton, and Greg Schneider: Texas Natural History Collection, Texas Memorial Museum, Austin; Texas Cooperative Wildlife Collection, College Station; Mayborn Museum Complex, Baylor University, Waco; and Museum of Zoology, University of Michigan, Ann Arbor.

Year	Institution	ID	Collector	Locality	Prep	Reference
Austin County						
	unknown	9 specimens		near Sealy		Sanders 1953
1990	unknown		Yantis	~8 km E of New Ulm on FM1094, N of roadway, upper end of Swenson Lake	sound recording	Price 1990b, Yantis 1990
Bastrop County						
	TCWC	87318	Forstner		ethanol	
	TCWC	90751-90752	Swannack	GLR		
	TCWC	70741, 70758	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
	TCWC	70742	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
	TCWC	70743	HZG	3.3 mi N entrance to BSP, Hwy 21, 0.75 mi SE Hidden Lake (Jim Small Ranch)	ethanol	
	TCWC	70747, 70749, 70755	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
	TCWC	70791, 70794	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
	TCWC	70795-70797, 70801-70802, 70806	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
	TCWC	90303-90304	HZG			
	TNHC	34740, 35536-35537		Bastrop		Potter et al. 1984

Year	Institution	ID	Collector	Locality	Prep	Reference
1951	TCWC	80731	Osborne	2.5 mi NE Bastrop, off Hwy 21	ethanol	
1963	TCWC	70807-70817, 70836-70838, 70840-70864	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
1963	TCWC	70865, 70870	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
1963	TCWC	70869	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
1966	TNHC	50117	Brown	BSP, 2 mi SE in Barking Dog Pond		
1966	TNHC	62425			skeleton	
1967	MVZ	81946-81947	Sage	BSP	ethanol	
1968	TCWC	82097	Quinn	4 mi N, 1 mi W Bastrop, BSP entrance off Hwy 21	skeleton	
1970s	TCWC	80729-80730	HZG	2.5 mi NE Bastrop, off Hwy 21	ethanol	
1971	MVZ	99123-99125	Sweet	0.5 mi SE BSP	ethanol	
1978	TCWC	70542, 70544-70560, 70566-70598, 70604, 70607, 70609-70611, 70614, 70623-70631	HZG	6.44 km N BSP entrance, 1.61 km W	larval and embryo	
1978	TCWC	70599-70603, 70605-70606, 70608, 70612-70613, 70615, 70618-70620, 70632-70633, 70648	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
1979	TCWC	70543, 70561-70565, 70616-70617, 70621-70622	HZG	5.47 km N BSP entrance, Hwy 21, 2.4 km E	larval and embryo	
1979	TCWC	70634-70647, 70649-70704	HZG	5.47 km N BSP entrance, Hwy 21, 2.4 km E	ethanol	
1979	TCWC	72908	HZG	5.49 km N BSP, 2.4 km E Hwy 21	ethanol	
1979	TNHC	50622	Hillis & Mosier	in pond 2.6 mi from BSP entrance on Hwy 21		
1980	KU	190153-190154		no data	ethanol	Thomas & Dessauer 1982
1980	TCWC	60035-60036			ethanol	Thomas & Dessauer 1982
1980	TCWC	70705-70711, 70726-70727, 70733, 70735-70736, 72907	HZG	3.3 mi N BSP entrance, Hwy 21, 1 mi E, permanent pond	ethanol	
1980	TCWC	70712-70725, 70728-70732, 70734	HZG	3.3 mi N BSP entrance, Hwy 21, 1 mi E, permanent pond	larval and embryo	
1980	TCWC	70818-70825, 70828-70834	HZG	3.3 mi N BSP entrance, Hwy 21 (Jim Small Ranch), 1 mi E (across from power line)	ethanol	
1981	TCWC	70785-70786	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
1981	TNHC	49375-49379	Hillis	BSP entrance, 5 km NE along Hwy 21; site 4, at roadside pond: 'Big Fence'		Hillis et al. 1984
1981	TNHC	49380	Hillis	BSP entrance, 7 km NE along Hwy 21; site 3, at roadside pond: Trailer Pit Pond'		Hillis et al. 1984

Year	Institution	ID	Collector	Locality	Prep	Reference
1981	TNHC	49381-49383	Hillis	BSP entrance, 4 km NE along Hwy 21; site 5, at roadside pond: '4 Score Pond'		Hillis et al. 1984
1981	TNHC	49384-49392	Hillis	BSP entrance, 2 km NE along Hwy 21; site 6, at roadside pond: 'Bog Pond'		Hillis et al. 1984
1981	TNHC	49394	Hillis	BSP entrance, 4 km NE along Hwy 21; site 5, at roadside pond: '4 Score Pond'		Hillis et al. 1984
1981	TNHC	49395	Hillis	FM 1441, 4 km NW jct Hwy 21; site 2, at roadside pond: 'Twin Ponds'		Hillis et al. 1984
1982	TCWC	60605	Dixon	2.5 mi W Hwy 21, Hwy 1441	ethanol	
1982	TCWC	60679, 60683	Dixon & McCrystal	0.6 mi NW Hwy 21, Hwy 1441	ethanol	
1982	TCWC	60680	Dixon & McCrystal	BSP	ethanol	
1982	TCWC	60681	Dixon & McCrystal	2.4 mi NW Hwy 21, Hwy 1441	ethanol	
1982	TCWC	60682	Dixon & McCrystal	2.2 mi N Bastrop	ethanol	
1983	TCWC	70740, 70745, 70754, 70764	HZG	3.3 mi N entrance to BSP, Hwy 21, 0.75 mi SE Hidden Lake (Jim Small Ranch)	ethanol	
1983	TCWC	70744, 70751-70753, 70762, 70766, 70768, 70773, 70783-70784	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
1983	TCWC	70748, 70750, 70787, 70826-70827, 70835, 70839, 70866-70868	HZG	3.3 mi N entrance BSP, Hwy 21, 1 mi E (study site C)	ethanol	
1983	TCWC	82098	Quinn	4 mi N, 1 mi W Bastrop, BSP entrance off Hwy 21	skeleton	
1987	TCWC	70756	HZG	no data	ethanol	
1988	TCWC	70757, 70774, 70777	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
1988	TCWC	70763, 70772, 70775	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
1988	TCWC	70765	HZG	3.6 mi N Hwy 21, Chapman Ranch, Houston Zoo 1980 study site B.	ethanol	
1988	TCWC	70767	HZG	3.3 mi N entrance to BSP, Hwy 21, 0.75 mi SE Hidden Lake (Jim Small Ranch)	ethanol	
1988	TCWC	70769-70770, 70778-70780, 70789-70790, 70792-70793	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
1988	TCWC	70771, 70781-70782, 70788, 90302	HZG	no data	ethanol	
1988	TNHC	64553-64554	Hillis & Cocroft	TX rte 21, 0.8 km SW jct FM	alcohol	
1989	TCWC	67799-67800	Dixon	BSP, 2 mi S Bastrop	ethanol	
1989	TCWC	70737-70739	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
1989	TCWC	70798-70800	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
1990	BLB	17445	Benson	BSP	sound recording	
1990	TCWC	67563-67566	Price	BSP, study pond #10	ethanol	Price 1990b

Year	Institution	ID	Collector	Locality	Prep	Reference
1990	TCWC	67898	Whiting	Hwy 21, 1.3 mi, plus 29 yards E BSP, S side	ethanol	Price 1990a
1990	TCWC	68210	Whiting	Hwy 21 (S side), 4.4 mi plus 15 yds E BSP	ethanol	Price 1990a
1990	TCWC	68214	Whiting	Hwy 21, 3.85 mi E BSP, N side	ethanol	Price 1990a
1990	TCWC	68255, 68258	Whiting	Hwy 21, 1.3 mi plus 20 yd E BSP, S side	ethanol	Price 1990a
1990	TCWC	68256	Whiting	Hwy 21 (S side), 4.6 mi E BSP	ethanol	Price 1990a
1990	TCWC	68257	Whiting	Hwy 21, 0.6 mi, 23 yards E BSP, S side	ethanol	Price 1990a
1990	TCWC	68259	Whiting	Hwy 21, 2.1 mi E BSP, N side	ethanol	Price 1990a
1990	TCWC	69812	Murray	4.1 mi - 60 yds E BSP entrance on S side of Hwy 21	ethanol	Price 1990a
1990	TCWC	69813	Murray	4.3 mi E BSP entrance on N side of Hwy 21	ethanol	Price 1990a
1990	TCWC	69815	Murray	1.3 mi E BSP entrance on N side Hwy 21	ethanol	Price 1990a
1990	TCWC	69816	Murray	3.1 mi - 38 yds E BSP entrance, S Hwy 21	ethanol	Price 1990a
1990	TCWC	70804-70805	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
1991	TCWC	70759	HZG	3.6 mi N Hwy 21, Chapman Ranch, Houston Zoo 1980 study site B.	ethanol	
1991	TCWC	70803	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
1991	TNHC	55492-55516	Price	BSP, pond #5	alcohol	Price 1992
1992	TCWC	82099	Quinn	4 mi N, 1 mi W Bastrop, BSP entrance off Hwy 21	skeleton	
1993	TCWC	70376	TXDPH	Hwy 21 1.5 mi E FM 1441	ethanol	
1993	TCWC	71657	Dixon	0.3 mi S Hwy 71 on Co Rd 191, then 1 mi E on gravel road	ethanol	
2003	TCWC	87316-87317	Forstner		ethanol	
2004	TCWC	90257-90261	Forstner	GLR		
2004	TCWC	90753-90754	Swannack	GLR		
2005	TCWC	90736	Jones	BSP, burn area		
Brazos County						
1962	MSUM	HE.8877	Schwille	4.2 mi. NE of Peach Creek community, along dirt road	fluid	
Burleson County						
1950	TCWC	7068-7069	Robertson	4 mi N Caldwell	ethanol	Sanders 1953
1989			Yantis	3.3 mi SW of Caldwell via TX Hwy 21 to jct with RR908, then ~2 mi N to Cade Lakes		Price 1990b
Colorado County						
	unknown	7 specimens		6 mi E Columbus		Sanders 1953
	SM	92-a-32-41		6 mi E Columbus	wet	Sanders 1953
	UMMZ	127826		6 mi E Columbus		Sanders 1953
1982	TCWC	62388	King	Attwater Prairie Chicken National Wildlife Refuge	ethanol	
1990	unknown		Yantis	~9 km S of New Ulm via county rds, 200 m E of county rd & just N of Hayes Creek	sound recording	Price 1990b, Yantis 1990
1990	unknown		Yantis	~4 km S of Frelsburg by TX Hwy 109 & ~4 km E by county rd on N side of E fork of a small creek, 150-200 m NE of county rd	sound recording	Price 1990b, Yantis 1990

Year	Institution	ID	Collector	Locality	Prep	Reference
Freestone County						
1990	unknown		Yantis	~8 km S & 5 km E of Lanely by county rds, E side of county rd & E side of triangle driveway		Price 1990b, Yantis 1990
Harris County						
	FLMNH	12948	Tabony			
	FMNH	74725	Wottring			
	LACM	87721	Hansaker, Giht, & Blair	Houston		
	TCWC	70746, 70760-70761	HZG	HZG	ethanol	
	TNHC	34741		Houston		Potter et al. 1984
1949	SM	16807-16808	Wottring	East Haven	wet	Potter et al. 1984
1950	CM	29172	Wottring	Fairbanks	alcohol	
1950	CU	5538	Wottring	Houston	fluid	
1950	SM	16833	Wottring	East Haven	wet	Potter et al. 1984
1950	SM	16809-16815	Wottring	Fairbanks	wet	Potter et al. 1984
1950	SM	16816-16817, 16819, 16821-16832	Wottring	New Haven	wet	Potter et al. 1984
1950	SM	16818, 16820	Wottring	vicinity of Houston	wet	Potter et al. 1984
1950	TNHC	28860	Wottring			
1950s	TCWC	80724-80728	Wottring	Houston	ethanol	
1951	CU	5499	Wottring	NW of Houston, Fairbanks	fluid	
1951	USNM	542212	Blair	1 mi S of Houston airport	ethanol	
1952	CAS	12768-12769*	Wottring & Greer	Fairbanks		Sanders 1953
1952	CM	32688-32690*	Wottring & Greer	Fairbanks	alcohol	Sanders 1953
1952	MCZ	A-28019 to A-28022*	Wottring & Greer	Fairbanks	alcohol	Sanders 1953
1952	SDNHM	42043-42044, 42049-42050		no data		
1952	SM	87-a-98-394 to 87-a-98-403*	Wottring & Greer	Fairbanks	skeletons	Sanders 1953
1952	UCM	11924	Wottring			
1952	UIMNH	33687-33689*	Wottring & Greer	Fairbanks		Sanders 1953
1952	UMMZ	127825	Wottring & Greer	Fairbanks		Sanders 1953
1952	UMMZ	127827	Wottring			
1952	USNM	134433-134436*	Wottring & Greer	Fairbanks	ethanol	Sanders 1953
1952	USNM	542211	Blair	Fairbanks	ethanol	
1958	TNHC	25626-25630	Wottring	Houston		Potter et al. 1984
1959	CM	63376	Wottring	Houston	alcohol	
1959	LSU	9307-9313	Fox & Wottring		isopropanol	
1988	TCWC	70776	HZG	HZG	ethanol	
1989	TCWC	70539-70540	HZG	HZG	larval and embryo	
1989	TCWC	70541	HZG	HZG	ethanol	

Year	Institution	ID	Collector	Locality	Prep	Reference
Houston County						
1970	TTU	5080-5082	Wade	Ratcliff Lake Reservoir	alcohol	
Lavaca County						
1991	TNHC	56005	Lehman	Hallettsville, SE at Upper Laughlins Sandy Creek in pond	alcohol	Yantis 1991
Lee County						
2001	TCWC	84556	Dixon	0.3 mi E Bastrop/Lee Co. line on County rd 333	ethanol	Gaston et al. 2001
Leon County						
1989	unknown		Yantis	pond 50-100 m W of Cherokee Lake		Yantis 1989, Price 1990b
1989	unknown	2 specimens	Yantis	water filled depression ~10 m E of Cherokee Lake		Yantis 1989, Price 1990b
1989	unknown		Yantis	water filled depression in RV park		Yantis 1989
1990	unknown	3 specimens	Yantis	trailer park E of Cherokee Lake		Price 1990b
1990	TCWC	68265-68270	Yantis	Hilltop Lake	ethanol	
1991	TNHC	55580-55590	Yantis	Hilltop Lakes Estates, vicinity of Cherokee Lake	alcohol	
Liberty County						
1950s	unknown		Gottsch	6 mi S Liberty		Sanders 1953
Milam County						
1987	TCWC	65498		6.2 mi SW Rockdale	ethanol	
Robertson County						
1975	TCWC	53989-53990	Thornton	10.0 mi WSW of Wheelock, EG Marsh farm	ethanol	
1989	unknown		Yantis	water filled sand pit, ~6 mi E Hearne on TX Hwy 391 then just N of Hwy on two-track		Yantis 1989, Price 1990b
2000	TCWC	84246	Hibbitts	5.6 mi W Jct FM 46/391 on 391	ethanol	
Travis County						
1952	INHS	6373	Smith & Minton			
Unknown						
	TNHC	60459, 60722			alcohol	
	TNHC	60879			skeleton	
1961	TNHC	62426			skeleton	
1969	LSU	47849		Sam Houston State Park, Houston Co.?	isopropanol	

For references, see Chapter 1.

VITA

Diana Jane McHenry was born on February 3, 1974 in Brunswick, Maine. She graduated from Great Bridge High School, Chesapeake, Virginia, in 1992. As an undergraduate, Diana worked for Drs. Lytton Musselman and Rebecca Bray on *Isöetes* (quillwort) systematics and earned a B.Sc. in biology from Old Dominion University, Norfolk, Virginia in May 1997. She continued her work as a botany student with Dr. Michael R. J. Forstner at Texas State University, San Marcos, Texas, researching molecular systematics within the four o'clock family (Nyctaginaceae), and graduated with a M.Sc. in biology from Texas State University, in December 2002. Following a brief stint at the University of Texas Medical Branch-Galveston as a Research Associate for Dr. Johnny W. Peterson in the Department of Microbiology & Immunology, Diana enrolled at the University of Missouri-Columbia in August 2003. Now working in anuran conservation genetics, Diana earned her Ph.D. from the Division of Biological Sciences in May 2010.