Section 6 (Texas Traditional) Report Review

Attachment to letter dated NOV 03 2009

TPWD signature date on report August 31, 2009

Project Title: Houston toad metapopulation assessment and genetics: data necessary for effective recovery strategies in a significantly fragmented landscape

Final or Interim Report? Final

Grant #: E-76

Reviewer Station: Austin ESFO

Lead station was contacted and concurs with the following comments:
☐ Yes ☐ No ☒ Not applicable (reviewer is from lead station)

.....

Interim Report (check one):	**Final Report** (check one):
☐ is acceptable as is	☐ is acceptable as is
☐ is acceptable as is, but comments below need to be addressed in the next report	☒ is acceptable, but needs minor revision (see comments below)
☐ needs revision (see comments below)	☐ needs major revision (see comments below)

Comments:

On page 3, the report refers to "a pending County level Habitat Conservation Plan." This Habitat Conservation Plan was finalized in April 2008. The report should be revised to reflect this.

Please clearly indicate on a map where the 9 discernable populations are (i.e., the populations referred to on page 77) and provide the X,Y coordinates and projections for these sites as well.

FINAL REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E – 76 -R

Endangered and Threatened Species Conservation

Houston Toad Meta-population Assessment and Genetics: Data Necessary for Effective Recovery Strategies in a Significantly Fragmented Landscape

Prepared by:

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Executive Director

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31 August 2009

FINAL REPORT

STATE: ____Texas_____ **GRANT NUMBER:** ___TX E-76-R_____

GRANT TITLE: Houston Toad Meta-population Assessment and Genetics: Data Necessary for Effective Recovery Strategies in a Significantly Fragmented Landscape

REPORTING PERIOD: ____1 Aug 06 to 31 Aug 09_

OBJECTIVE(S):

To evaluate the Houston toad range-wide status including meta-population genetics useful in current management strategies and conservation plans.

Significant Deviation:

None

Summary Of Progress:

Please see Attachment A.

Location: Austin, Bastrop, Colorado, Lavaca, Lee, Liberty, and Milam Counties, TX

Cost: ___Costs were not available at time of this report, they will be available upon completion of the Final Report and conclusion of the project.__

Prepared by: _Craig Farquhar_____ **Date:** _31 August 2009

Approved by: _____ **Date:**_____
C. Craig Farquhar

Final Report

Houston toad metapopulation assessment and genetics: Data necessary for effective recovery strategies in a significantly fragmented landscape

Section 6 #E-76

Submitted to

Texas Parks and Wildlife Department

&

the United States Fish and Wildlife Service

by

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Introduction

The Houston toad (*Bufo houstonensis*) persists on the landscape in widely scattered populations among which isolation and consequent population interactions vary at several spatial scales. The species is ecologically adapted to local extirpation events and subsequent recolonization is believed to be a normal part of the metapopulation cycle. The Houston toad also has a tremendous reproductive potential, thus providing a remarkable innate capacity for population growth and eventual recovery. As a conservation initiative, the current situation for the Houston toad is critical and the situation is currently even more dire given the ongoing drought severity and impacts. In the last five years, stakeholder efforts focusing on the toad have become focal to the Bastrop County community. As a result, private landowner Safe Harbor agreements, newly purchased Conservation Lands, and a pending County level Habitat Conservation Plan have been incorporated toward Houston toad recovery.

This has coincided with renewed regional-scale research efforts both within Bastrop County (Forstner 2002; 2003) and in adjacent Lee County (Forstner and Dixon 2001). However, most of the current and recently completed research efforts have been focused on habitat use and ecology of the species centered on the scale of ponds and inter-pond movements (Forstner and Swannack 2004). Our own ongoing research seeks to evaluate land use and habitat restoration as means toward recovery of the species in Bastrop County (Forstner 2004). Those efforts are again at a local, single forest fragment scale. If we examine the historic scientific knowledge base for the Houston toad, we find that data primarily exist for two broad areas: distribution and life history. While historical survey data provide distributional evidence (Yantis 1989-1992), nearly all other work on the toad has been at the pond or county scale (Price 2003). Yet, while all of the previous and ongoing research avenues have been guided by the Houston toad Recovery Plan (1984), range-wide biological data remain as important as in 1984 (USFWS 1984) and as unknown today as it was then.

The 1984 Recovery Plan specified range-wide surveys as a primary goal of immediate need in 1984, and Yantis (1989-1992) completed such audio chorus surveys. Since that time, the data from ponds on Bastrop State Park show a dramatic decline in the toads during the 1990s with some evidence of stabilization during the years representing

the end of the study (ie. 2002) (Price 2003). In this recent period, surveys that sought data at the county-wide scale have been completed for both Bastrop (Forstner 2002) and Lee Counties (Forstner and Dixon 2001). Beyond those two counties however, virtually no current data exist evaluating chorusing in other historically documented locations. This is a particular concern as many of the locations outside of Bastrop and Lee County are likely to represent very small populations with low numbers of individuals compared to the Houston toads of Bastrop State Park. Those smaller populations are thus at higher risk of extirpation during episodes like the drought of the 1990s and may not be recolonized (Blaustein et al. 1993).

Population genetic data is also missing from the underlying dataset used by management and stewardship efforts. Population genetic data has the potential to reveal far more than simply a given occurrence but the historical connectivity, population diversity, and evolutionary context of the Houston toad. However, no samples of Houston toad tissue are archived at any facilities other than our own. Indeed, the lack of historical samples has only increased the need for an assessment now, before any further regional extirpation has occurred. Thus, the persistence of Houston toads outside of Bastrop County is unknown and the historical connectivity of those populations to Bastrop County is likewise unknown.

Ultimately, this is a problem for management, as all current thinking about the Houston toad reflects the idea that extinction will be prevented only if conservation efforts focus on metapopulation dynamics (Hatfield et al. 2004). Unfortunately, we have virtually no information at this spatial scale that can help to guide management strategies and conservation efforts. Preventing extinction of the Houston toad over the next several decades is only possible if groups of populations are able to act as fluctuating reservoirs for recolonization as local extirpation of population subsets occur (Hatfield et al. 2004).

Objective: Evaluate the Houston toad range-wide status and conduct genetic analyses useful for implementing sound management strategies and effective conservation plans.

Methods and Materials:

Audio survey methods

Audio surveys for occupied or historically occupied regions provide us with the ability to compare the data with the historical survey database for the toad. The collection of the survey data met or exceeded the guidelines specified currently by the USFWS for audio surveys of the Houston toad. Because many of the locations have not been visited for Houston toad surveys in more than ten years, we have also examined potential habitat adjacent to those specific locations, using the historic site as the starting reference for each region. Subsequently, we visited the localities, examining potential breeding sites and the characteristics of the habitat on a local scale to the historical locality. Then appropriate nightly conditions led to audio surveys of the localities and of listening survey routes were conducted. Chorus surveys for amphibian detection are fairly standardized and we have recently published an analysis of our methods which provides statistical evidence that we are unlikely to fail to detect Houston toads at a location should they occur at that pond (Jackson et al. 2006). The historical range for the Houston toad is not large on a geographic scale, but it is too large on a survey scale to enable annual rangewide work with any detail in coverage. Consequently, our approach was to survey a County, if Houston toads were detected in an area we then moved on to other areas. Thus, our efforts became quickly concentrated in those areas for which the populations with low detection probabilities.

While audio chorus survey results provide a snapshot of the occupancy status for a region, only population genetics can assist with the historical connectivity and ecology of this species. DNA sampling of Houston toads was be completed non-consumptively with genomic samples obtained from blood/toe during each of the surveys conducted. We have assembled blood samples from several localities using this method across the past ten years, routinely recapturing the sampled adults in subsequent chorus nights and years. As all of the samples taken are collected in the field, handling is tied to data recording methods and appropriate sterile technique. All samples are collected from living animals by sterile syringe or scissors and placed into sterile cryogenic storage tubes. In the event of any physical encounters for deceased individuals, tissue samples and appropriate voucher specimens were salvaged.

The laboratory work has standardized methods (Avise 1994, Smith and Wayne 1996) allowing us to carry on the laboratory work with as much confidence as is possible with population genetic studies. All laboratory work is confined to workspaces designed for such work. All work from the initial tissue sample collections through DNA extractions, amplification, and subsequent allele/base pair calling are strictly controlled with appropriate positive and negative controls. Peak height, signal to noise ratios, and size standard controls act to guide the precision of allele calls and accuracy of DNA base pair assignments from the automated sequencing platform. Accuracy of the hardware is specified by the manufacturer as less than 1% error rate, which is itself halved by our complete bidirectional sequencing of all templates.

Molecular methods

Sampling - Individuals were sampled across southeast-central Texas, the historical range of *B. houstonensis* (Appendix 1), from 2000 to 2008. 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; USFWS 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. Voucher 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.

DNA Sequences- A ~500 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 95ºC for 5 min then 35 cycles, each consisting of denaturing at 95ºC for 30 sec, annealing for 1 min (see Table 3 for annealing TºC for each locus), and extension at 72ºC for 1 min, and a final extension period of 72ºC 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.

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 (10000 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 burnin of a 2500 trees.

To assess intraspecific relationships, a statistical parsimony network (Templeton et al. 1992) of mtDNA haplotypes in *B. houstonensis* was constructed using TSC 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), 1000 stored iterations (1100000 iterations, 1000 thinning, 100 burnin) were used, the null allele model was not used, and the spatial model was used.

Guillot et al. (2005a) suggest 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 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 samples (97.3%) was 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 also included. Analyses were performed using these ten subsets, to determine K and then to assign individuals to clusters, as described above.

Since most *B. houstonensis* currently are found in Bastrop County (Hillis et al. 1984; USFWS 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, burnin was 100000, number of MCMC reps after burnin was 1000000, 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*. 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 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 samples (97.3%) was 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 also included. Analyses were performed using these ten subsets, to determine *K* and then to assign individuals to clusters, as described above.

Since most *B. houstonensis* currently are found in Bastrop County (Hillis et al. 1984; USFWS 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 1000000 Markov chain steps and 100000 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}s in ARLEQUIN version 3.11 (Excoffier et al. 2005) with 10000 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 10000 permutations each, ten distance classes (using Euclidian distances) and unequal class sizes:

1) all individuals ($n = 439$)
2) all individuals but with logarithm transformed geographic distances
3) only individuals from Bastrop and Lee counties ($n = 427$)

4) only individuals from Bastrop and Lee counties but with logarithm transformed geographic distances
5) only individuals from Bastrop County (n = 416)
6) only individuals from Bastrop County but with logarithm transformed geographic distances.

Four analyses were performed using the mtDNA sequence dataset: all individuals (n = 107), all individuals but with logarithm transformed geographic distances, only individuals from Bastrop County (n = 95), and only individuals from Bastrop County but with logarithm 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 = 3000000, burnin = 1000000, 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 = 3000000, burnin = 1000000, 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 strategie 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 large enough. 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 10000 permutations.

Results

Range wide surveys

2007: We conducted surveys that met or exceeded detection probabilities of 0.90 (Jackson et al., 2006) in the following counties during 2007: Austin, Bastrop, Colorado, Lavaca, Lee, Liberty, and Milam. We also revisited historical localities and performed less than 10 survey nights in the following counties in 2007: Burleson, Ft. Bend, Harris,

Limestone, Leon, Robertson. These visits were generally site assessment of habitat with canopy identified by aerials, or accompanying Dr. Jim Yantis during his review of both habitat and historical sites on his routes. Houston toads were found to be actively chorusing over time and in numbers greater than 10 individual males (total seasonal count) in only two counties (Bastrop and Milam). A single toad was found in Leon, a single toad was heard in Lee, three toads were found and two others heard in Colorado, but no significant chorusing was detected in any of the historical locations in any of these counties. This is particularly unfortunate given that significant choruses did occur in Lee County as recently as 2001. The two senior authors (MRJF and JRD) noted in a letter submitted to the USFWS in that year that significant unpermitted clearing on documented, occupied Houston toad habitat was being conducted. Those forests and along with them the toad is now gone from a significant portion of what was occupied in Lee County (Forstner and Dixon 2000; 2001).

2008: We conducted surveys which met or exceeded detection probabilities of 0.90 (Jackson et al. 2006) in the following counties during 2008: Austin, Bastrop, Colorado, Lavaca, Lee, and Milam. We also revisited historical localities and performed less than 10 survey nights in the following counties in 2008: Burleson, Leon, and Robertson. Finally, we extended our surveys into adjacent counties to those known to be occupied by Houston toads, or those with otherwise appropriate habitat; Anderson, Guadalupe, Henderson, and Wilson. Houston toads were found to be actively chorusing over time and in numbers greater than 10 individual males (total seasonal count) in only two counties (Bastrop and Milam). A single chorus of more than ten toads was heard one night in Leon, three toads were found and two others heard in Austin, but no significant chorusing was detected in any of the historical locations in any of these counties. At this time we consider the Houston toad to be likely extirpated in Lavaca County, unlikely to occur in Lee County, and at very low numbers in Austin, Colorado, Lee, and Leon counties.

2009: We conducted surveys which met or exceeded detection probabilities of 0.90 (Jackson et al. 2006) in the following counties during 2009: Austin, Bastrop, Colorado, and Lavaca. We also revisited historical localities and performed less than 10 survey nights in the following counties in 2009: Lee, Leon, Milam and Robertson.

Finally, we extended our surveys into adjacent counties to those known to be occupied by Houston toads, or those with otherwise appropriate habitat and completed less than 10 nights; Caldwell and Guadalupe. Houston toads were found to be actively chorusing over time and in numbers greater than 10 individual males (total seasonal count) in only two counties (Austin and Bastrop). The results from Austin County include more than ten toads likely at a single site in the County, not widespread large chorusing events. The vast majority of chorusing for 2009 occurred in a single weekend of mid April. This is consequent of drought and the one large rainfall episode for the spring of 2009. At this time we consider the Houston toad to be likely extirpated in Lavaca County, at the threshold for extirpation in Lee County, and at very low numbers in Austin, Colorado, and Leon counties.

The complete GIS layer of locations from which Houston toads were detected has been provided as a digital data layer to the Austin office, USFWS prior to the submission of this report.

Genetic analyses

Sampling

Four hundred thirty-nine *B. houstonensis* in six counties from 2000-2008 were sampled for this study (Fig. 1, Table 2). 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. not yet available)**. 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).

Microsatellites

Ten microsatellite loci have been shown to be homologous to published sequence and are suitably polymorphic in Houston toads (Table 3). Two loci have been tested thoroughly, and while they are homologous to published sequence and polymorphic, this polymorphism turned out to be a result of indels (insertion deletion events not related to the microsatellite locus itself) and thus not changes in number of microsatellite repeats: Bbuf15 (Brede et al. 2001) and BC60.37 (Chan 2007). Six loci amplified in Houston toads but were monomorphic: Bbuf49 (Brede et al. 2001), bco40 (Chan 2007), BM121, BM239 (Tikel et al. 2000), ICCC, and IDDD (Gonzalez et al. 2004). Ten loci amplified but were not microsatellite loci in Houston toads: BC52.03, BC52.04, BC52.11, BC60.20 (Chan 2007), BM128, BM217, BM229, BM279, BM322 (Tikel et al. 2000), and IKK (Gonzalez et al. 2004).

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 likelihood for K = 2 was –13505.1 and ΔK was 2023.9. 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 likelihood for $K = 3$ was –2454.8 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 X; 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 X. 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 likelihood for K was –13100.9 and ΔK 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 likelihoods for $K = 2$ were –4746.6 and –3959.8, 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}s 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; $Z = 1.45 < 1.9600$ 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; $Z = 2.64 > 1.9600$ 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,

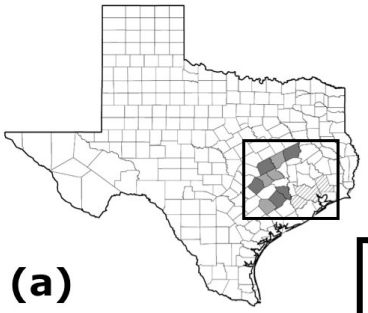
nine out of ten runs converged on similar solutions; data not shown). Migration rates from the best run are presented in Table 20; proportion of residents per group ranged 69.4%-93.6%. Standard deviations were mostly <0.05; twelve (out of 256) were between 0.051 and 0.080. Migration rates were generally 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.

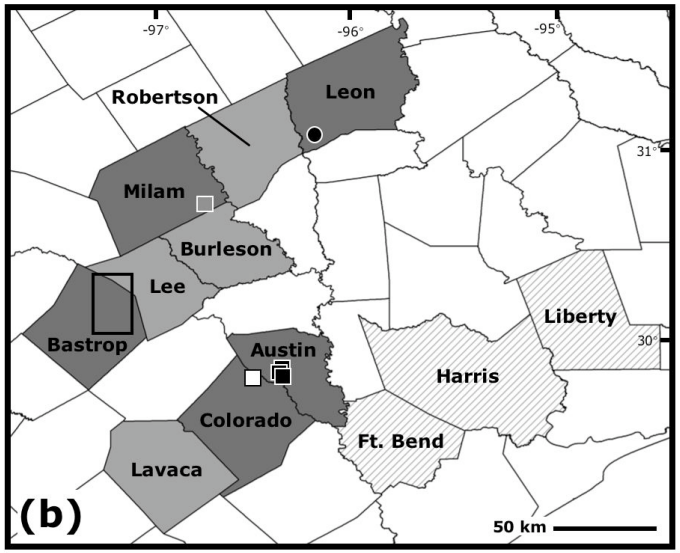
The proportion of males that were 'resident' was not significantly different from the proportion of females that were 'resident' in BANwest (H_0: proportion of males that were residents = proportion of females that were residents; $Z = 1.35 < 1.9600$ so fail to reject H_0), but the 95% CI indicated that 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; $Z = 2.17 > 1.9600$ so reject H_0; 95% CI = 0.097, 0.384).

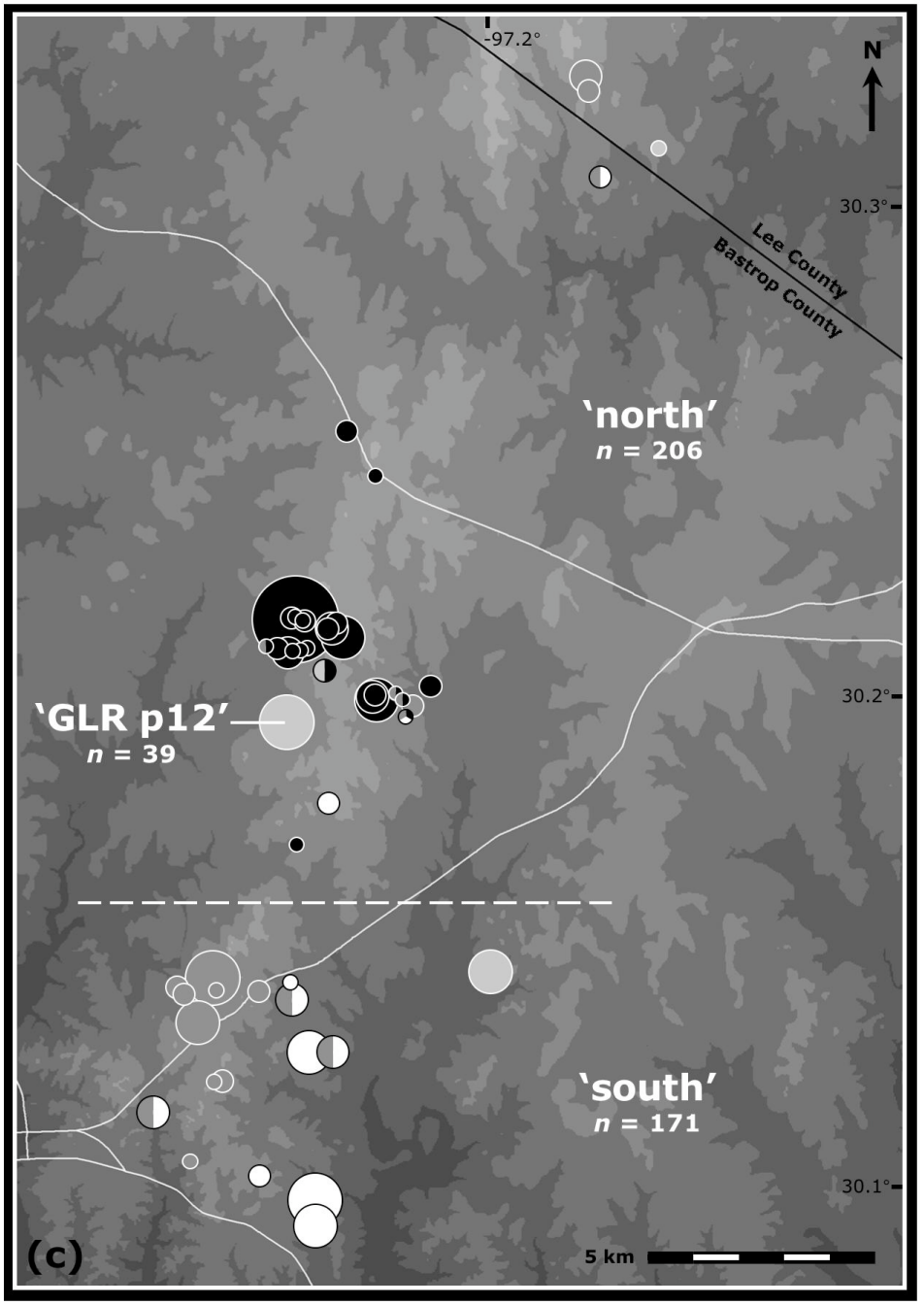
AMOVA analyses

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%).







Legend — (a) and (b)

Occurrence by county:
- extant
- not observed recently*
- extirpated
- never observed

Assignments:
- cluster I (*n* = 4)
- cluster N (*n* = 1)
- cluster S (*n* = 3)
- cluster U (*n* = 4, 3 sites)

Legend — (c)

Elevation (m):
- 200-220
- 180-200
- 160-180
- 140-160
- 120-140
- 100-120
- county line
- U.S. or State highway
- subgroup boundary

Assignments:
- cluster N
- cluster S_1
- cluster S_2
- cluster X
- $N+S_2$
- N+X
- $N+S_1+S_2$
- S_1+S_2

Sample size:
- 1
- 2-4
- 5-10
- 12-20
- 26-39
- 108

Map by Diana J. McHenry and Shawn F. McCracken

Fig. 1 (a) Occurrence of *B*. *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

Table 1. Number of Houston toad (*Bufo houstonensis*) tissues collected by county, locality, and specific locality.

County	Locality	Specific Locality	Number	Total Per Locality	Total Per County
Bastrop	Bastrop State Park	Melissa's traps	7	64	457
		BSP pond 11	11		
		BSP pond 19	20		
		BSP pond 8	26		
	Bluebonnet Headquarters	BBHQ pond 1	18	55	
		BBHQ pond 2	6		
		BBHQ pond 3	31		
	Bob Long	Bob Long Back Pond	19	19	
	Along 290	Dube Ln & Sandy Creek	5	10	
		Kuhl Site	4		
		Musgrave Pond	1		
	Griffith League Ranch	GLR unknown	9	265	
		GLR traps	54		
		GLR pond 10	3		
		GLR pond 11	4		
		GLR pond 12	39		
		GLR pond 15	2		
		GLR pond 2	111		
		GLR pond 3	2		
		GLR pond 5	7		
		GLR pond 6	5		
		GLR pond 7	11		
		GLR pond 8	4		
		GLR pond 9	13		
		Old Fire Tower Rd & 1441	1		
	Jim Small	JS pond 1	3	37	
		JS pond 2	5		
		JS pond 3	1		
		JS pond 4	20		
		JS pond 5	7		
		JS pond 6 (Jake's mudhole)	1		
	Unknown	Unknown	7	7	
Colorado	CR-52	CR-52, near intersection with Warsehak Schuette Rd	3	3	3
Lee	CR-333	CR-333, 2.7 mi S jct CR-331 & CR-333	1	1	19
	Durham	Durham pond 1	7	17	
		Durham pond 2	10		
	F3 pond 6	F3 pond 6	1	1	
Leon	Hilltop Lakes	Hilltop Lakes, Cherokee Lake	1	1	1
Milam	CR-342	CR-342	4	4	4
Unknown	Unknown	Unknown	3	3	3

Table 2 Numbers of Houston toad (*Bufo houstonensis*) individuals sampled from 2000 to 2008 per site by sex and geographic coordinates for each collection 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

Table 2 (cont.) Site	Latitude	Longitude	Male	Female	Unknown	Total
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 *B*. *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	#
	MF02968	NY: Orange Co.	unknown	MF02968	#
	MF07399	OK: Cleavand Co.	male	MF07399	#
	MF08153	MO: Taney Co.	male	wooD	#
	MF08154	MO: Taney Co.	male	MF08154	#
	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	#
	MF27040	TX: Randall Co.	juvenile	cogA	#
	MF27054	TX: Parmer Co.	unknown	cogA	
Bufo fowleri (*n* = 3)					
	MF05186	GA: Carroll Co.	unknown	MF05186	#
	MF10100	VA: Stafford Co.	female	fowA	#
	MF10103	VA: Stafford Co.	female	fowA	
Bufo woodhousii (*n* = 20)					
	MF03523	TX: Wichita Co.	juvenile	wooC	#
	MF05270	TX: Hill Co.	male	wooB	#
	MF05271	TX: Hill Co.	unknown	wooB	
	MF05272	TX: Hill Co.	unknown	wooA	#
	MF05273	TX: Hill Co.	unknown	wooB	
	MF05274	TX: Hill Co.	unknown	wooA	
	MF07398	OK: Cleavand Co.	male	MF07398	#
	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	
	MF21487	TX: Hill Co.	male	wooB	
	MF22054	TX: Aransas Co.	female	wooE	#
	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

Table 6 (cont.) BAYESASS Group	Site	GENELAND analysis A results	GENELAND analysis C results
	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

Table 8 (cont.)

	Site	2000-01[a]	2002	2003	2004	2005	2006	2007-08[b]	Total
	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

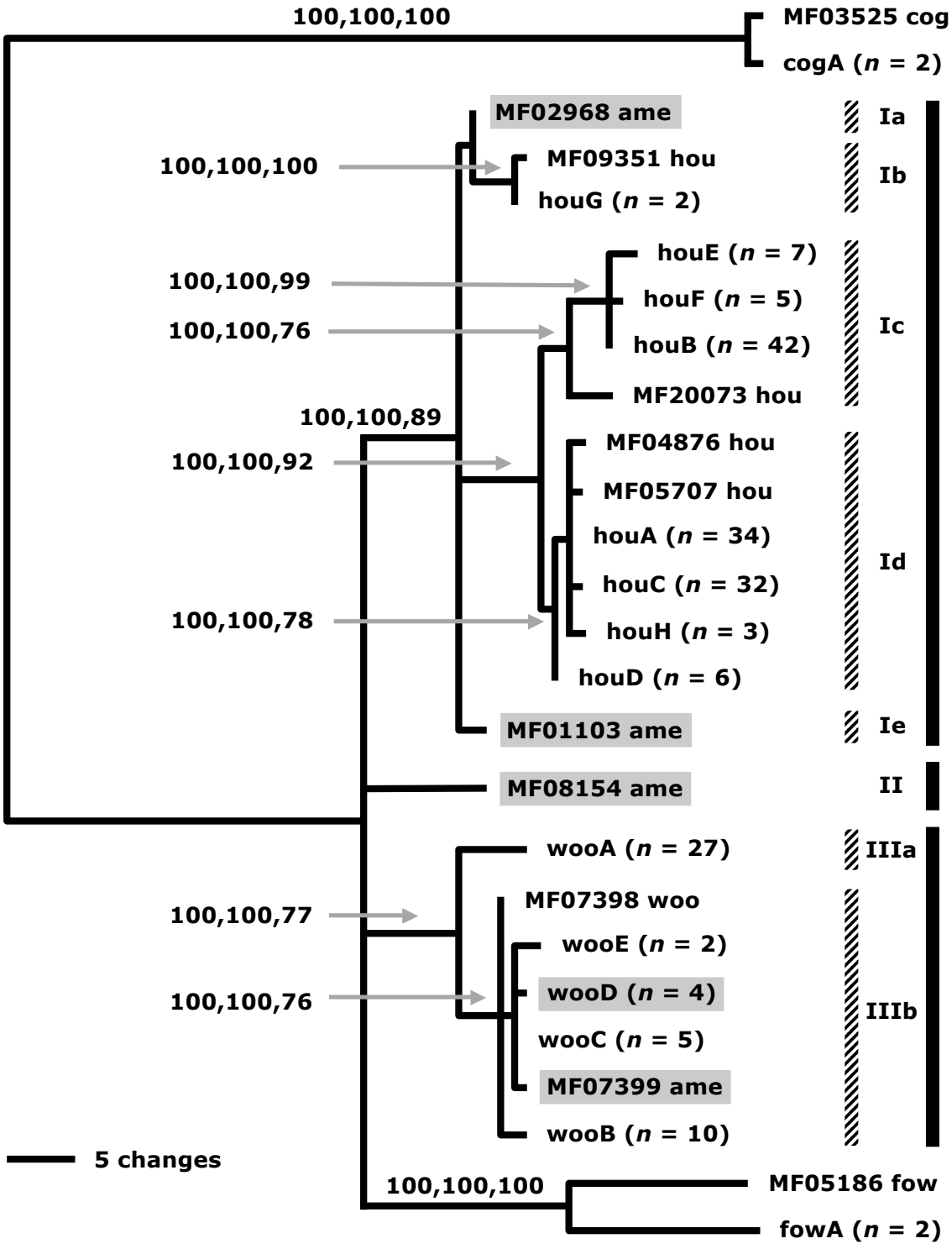

100,100,100
MF03525 cog
cogA (n = 2)
MF02968 ame
Ia
100,100,100
MF09351 hou
houG (n = 2)
Ib
houE (n = 7)
100,100,99
houF (n = 5)
100,100,76
houB (n = 42)
Ic
MF20073 hou
100,100,89
MF04876 hou
100,100,92
MF05707 hou
houA (n = 34)
houC (n = 32)
100,100,78
houH (n = 3)
houD (n = 6)
Id
MF01103 ame
Ie
MF08154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

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

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

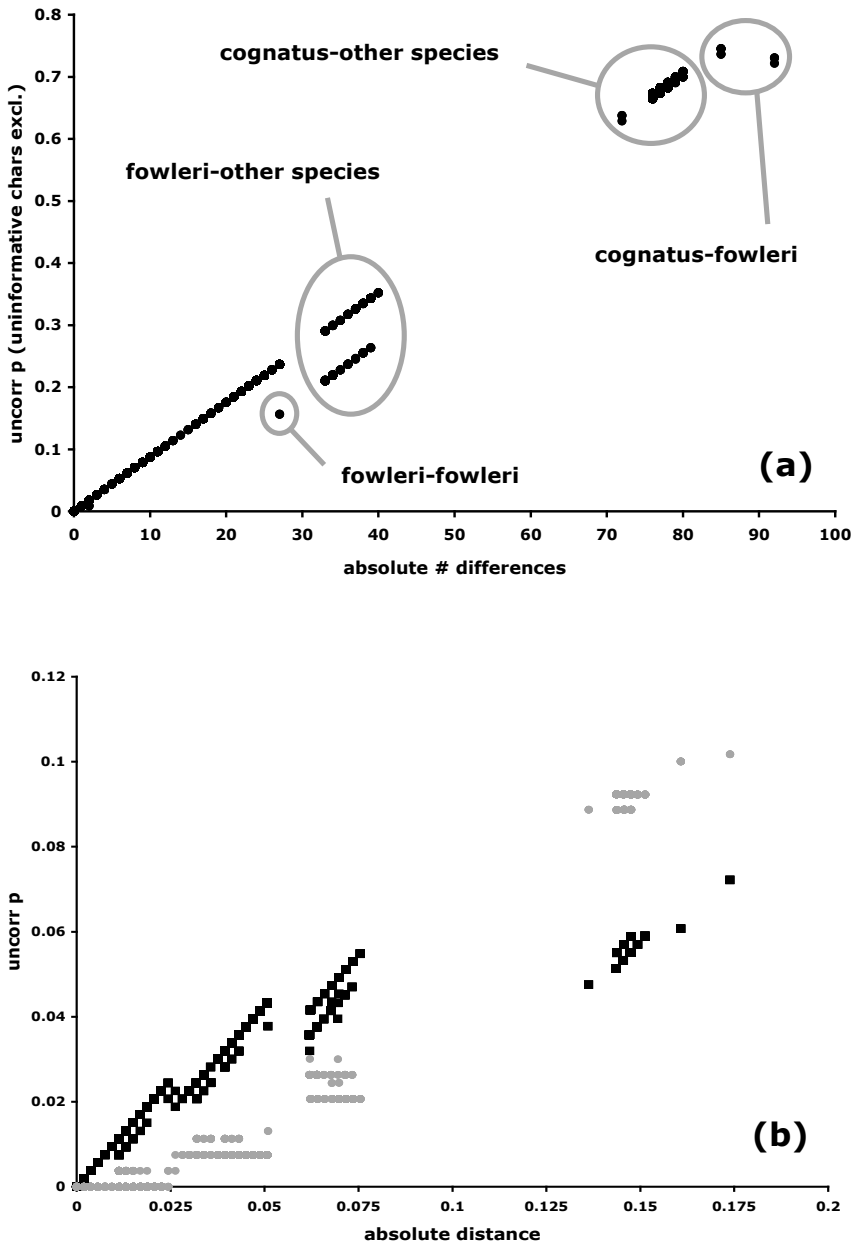


Fig. 3 (a) Uncorrected pairwise distance (after excluding uninformative characters) plotted against absolute number of differences. Pairwise comparisons of fowleri-fowleri, fowleri-other species, cognatus-fowleri, and cognatus-other species are indicated by grey circles. Data points not enclosed in a grey circle are comparisons within cognatus and among or within americanus, houstonensis, and woodhousii. Saturation is observable 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 is observable at distances >0.125 (pairwise comparisons involving cognatus)

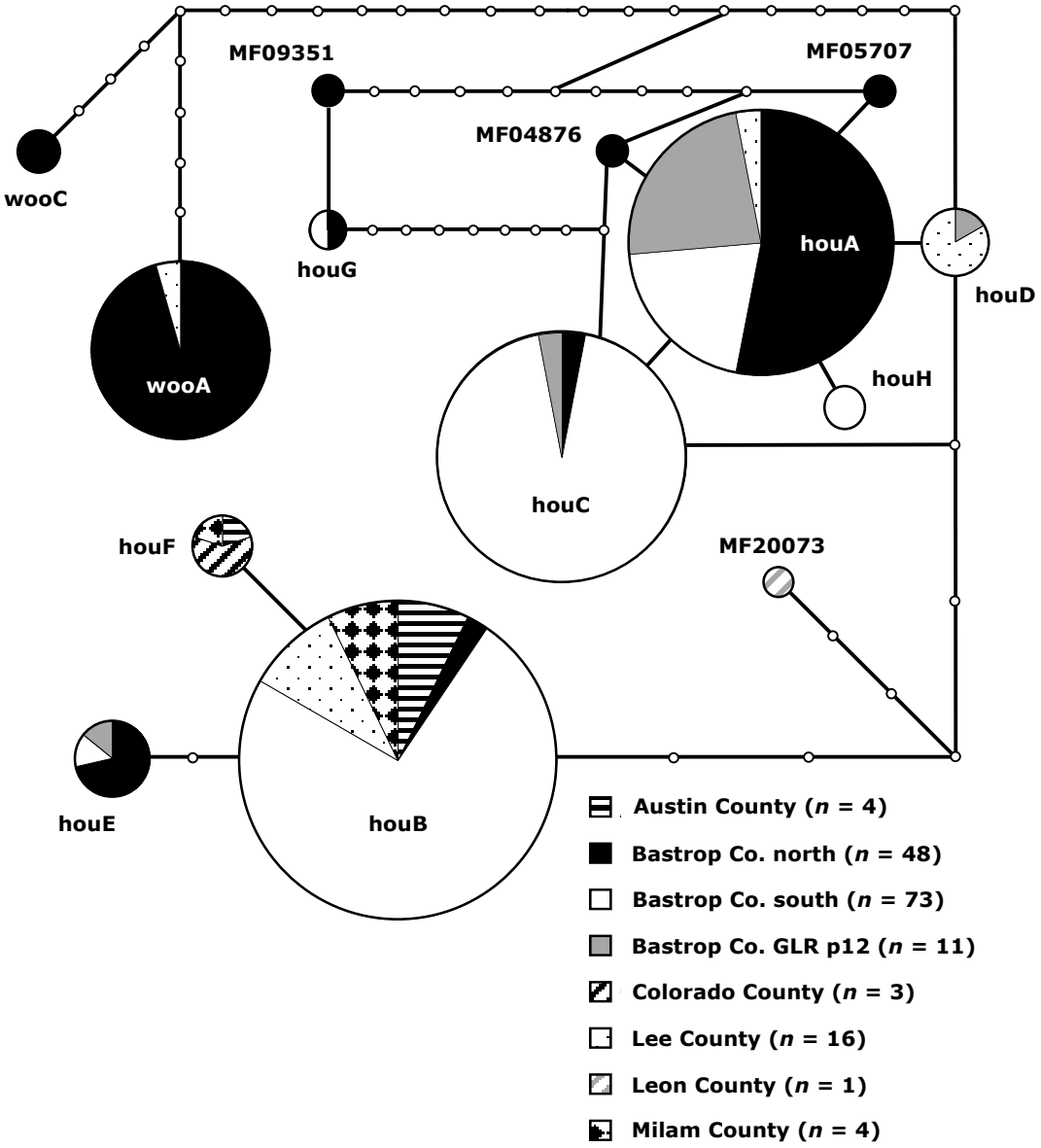


Fig. 4 Statistical parsimony network of 14 mtDNA haplotypes in 160 *B. houstonensis*. Circle size is proportional to number of individuals: houA (n = 34), houB (n = 42), houC (n = 32), houD (n = 6), houE (n = 7), houF (n = 5), houG (n = 2), houH (n = 3), MF04876 (n = 1), MF05707 (n = 1), MF09351 (n = 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

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

Table 11 (cont.)

		GENELAND analysis					
Site	*n*	A	B	C	D	E	F
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	—	N+S+X	N+S+X	S+X	—	S+X	—	S+X
*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, 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 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 *B. 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 natural logarithm 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
ln transformed geographic distance	0.1186	<0.0001
*Microsatellites, only Bastrop and Lee counties (*n = *427)*		
geographic distance (km)	0.1411	<0.0001
ln transformed geographic distance	0.1177	<0.0001
*Microsatellites, only Bastrop County (*n = *416)*		
geographic distance (km)	0.1039	<0.0001
ln transformed geographic distance	0.0973	<0.0001
*mtDNA, all individuals (*n = *107)*		
geographic distance (km)	0.1591	<0.005
ln transformed geographic distance	0.1488	<0.0001
*mtDNA, only Bastrop County (*n = *95)*		
geographic distance (km)	0.0938	<0.01
ln transformed geographic distance	0.0631	<0.01

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

	INTO																		
	Austin	Bastrop Co. north						Bastrop Co. south						BAPp	COLs	LEE01s	LEE02,03	LEOp	MILs
FROM		BAN09p	BAN27s	BANeast	BANnorth	BANsouth	BANwest	BAS06p	BAS08p	BAS15p	BAS18p	BASs1	BASs2						
Austin	**0.943**	0.012	0.011	0.002	0.011	0.011	0.001	0.001	0.008	0.011	0.009	0.000	0.001	0.002	0.012	0.012	0.002	0.012	0.012
BAN09p	0.003	**0.733**[a]	0.011	0.003	0.011	0.011	0.029	0.001	0.009	0.010	0.009	0.000	0.001	0.001	0.012	0.010	0.002	0.013	0.012
BAN27s	0.004	0.012	**0.722**	0.003	0.011	0.012	0.001	0.001	0.008	0.010	0.009	0.000	0.001	0.002	0.010	0.012	0.002	0.012	0.013
BANeast	0.003	0.012	0.023	**0.784**	0.054	0.012	0.070	0.001	0.008	0.013	0.009	0.000	0.001	0.005	0.013	0.013	0.002	0.011	0.014
BANnorth	0.003	0.012	0.011	0.003	**0.713**	0.012	0.001	0.001	0.008	0.011	0.008	0.000	0.001	0.002	0.012	0.011	0.002	0.011	0.011
BANsouth	0.003	0.012	0.011	0.003	0.010	**0.733**[a]	0.001	0.001	0.008	0.012	0.008	0.000	0.001	0.002	0.012	0.012	0.002	0.011	0.013
BANwest	0.003	0.034	0.015	**0.152**	0.056	0.022	**0.890**	0.001	0.017	0.021	0.018	0.000	0.002	0.029	0.017	0.012	0.002	0.016	0.018
BAS06p	0.003	0.011	0.012	0.003	0.013	0.012	0.001	**0.982**	0.008	0.037	0.010	0.000	0.001	0.003	0.013	0.017	0.002	0.012	0.012
BAS08p	0.004	0.013	0.011	0.003	0.011	0.011	0.001	0.001	**0.694**	0.010	0.008	0.000	0.001	0.002	0.011	0.011	0.002	0.011	0.013
BAS15p	0.003	0.012	0.011	0.002	0.010	0.014	0.001	0.001	0.008	**0.715**	0.010	0.000	0.001	0.002	0.012	0.011	0.002	0.013	0.012
BAS18p	0.003	0.011	0.011	0.002	0.012	0.012	0.001	0.001	0.009	0.010	**0.706**	0.000	0.001	0.002	0.012	0.012	0.002	0.012	0.029
BASs1	0.003	0.016	0.037	0.014	0.010	0.040	0.001	0.001	**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	0.004	0.040	0.011	0.004	0.011	0.013	0.001	0.001	0.052	0.014	0.045	0.000	**0.927**	0.002	0.036	0.012	0.002	0.014	0.012
BAPp	0.003	0.012	0.013	0.009	0.011	0.026	0.001	0.001	0.013	0.012	0.021	0.000	0.009	**0.934**	0.023	0.015	0.002	0.012	0.039
COLs	0.003	0.011	0.010	0.003	0.011	0.011	0.001	0.001	0.009	0.011	0.009	0.000	0.001	0.002	**0.731**[a]	0.011	0.002	0.013	0.011
LEE01s	0.003	0.011	0.012	0.003	0.011	0.011	0.001	0.001	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	0.003	0.012	0.046	0.003	0.011	0.011	0.001	0.001	0.010	0.012	0.012	0.000	0.001	0.001	0.012	**0.113**	**0.963**	0.015	0.013
LEOp	0.003	0.012	0.012	0.003	0.011	0.012	0.001	0.001	0.008	0.011	0.009	0.000	0.001	0.002	0.012	0.013	0.002	**0.772**[a]	0.013
MILs	0.003	0.012	0.010	0.003	0.012	0.013	0.001	0.001	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 *B. houstonensis* groups in BANwest described in Table 7, obtained using BAYESASS version 1.3

	INTO															
	sites near BAN02p					sites near BAN04p						sites near BAN05p				
FROM	BAN02p	BAN12t	BAN13t	BAN14t	BAN23t	BAN03s	BAN04p	BAN15t	BAN24t	BAN25t	BAN26t	BAN05p	BAN06p	BAN16t	BAN17t	BAN07p
BAN02p	**0.819**	0.022	0.056[b]	0.018	0.023	0.015	0.015[a]	0.016	0.014	0.015	0.046	0.087[a]	0.033[a]	0.039	0.021	0.058
BAN12t	0.001	**0.733**[b]	0.015	0.014	0.013	0.015	0.004	0.016	0.015	0.012	0.014	0.010	0.008	0.015	0.016	0.014
BAN13t	0.001	0.014	**0.733**[b]	0.014	0.014	0.014	0.003	0.013	0.014	0.015	0.013	0.009	0.007	0.014	0.016	0.014
BAN14t	0.001	0.015	0.015	**0.775**[b]	0.014	0.015	0.003	0.014	0.014	0.013	0.014	0.010	0.009	0.015	0.014	0.013
BAN23t	0.001	0.014	0.016	0.014	**0.777**[b]	0.013	0.004	0.015	0.014	0.015	0.014	0.010	0.008	0.015	0.014	0.013
BAN03s	0.001	0.014	0.014	0.016	0.014	**0.778**[b]	0.004	0.016	0.013	0.015	0.013	0.009	0.008	0.014	0.015	0.014
BAN04p	0.079	0.044	0.017	0.021	0.014	0.024	**0.936**[b]	0.023	0.026	0.023	0.058	0.058	**0.167**[ab]	0.021	0.051	0.047
BAN15t	0.001	0.014	0.015	0.014	0.015	0.015	0.004	**0.774**[b]	0.015	0.013	0.013	0.010	0.009	0.013	0.015	0.013
BAN24t	0.001	0.016	0.016	0.015	0.016	0.015	0.003	0.014	**0.776**[b]	0.014	0.014	0.010	0.008	0.013	0.015	0.012
BAN25t	0.001	0.014	0.014	0.013	0.014	0.014	0.003	0.015	0.013	**0.781**[b]	0.013	0.009	0.008	0.014	0.015	0.014
BAN26t	0.001	0.014	0.016	0.016	0.015	0.015	0.003	0.015	0.015	0.014	**0.720**	0.010	0.008	0.017	0.015	0.013
BAN05p	0.001	0.013	0.014	0.015	0.014	0.013	0.003	0.013	0.013	0.013	0.014	**0.702**	0.008	0.015	0.015	0.014
BAN06p	0.065	0.033	0.015	0.012	0.014	0.013	0.005	0.014	0.015	0.015	0.013	0.039	**0.694**	0.015	0.016	0.014
BAN16t	0.023	0.015	0.016	0.015	0.013	0.015	0.004	0.014	0.014	0.014	0.014	0.010	0.008	**0.749**[b]	0.015	0.014
BAN17t	0.001	0.014	0.015	0.014	0.016	0.013	0.003	0.014	0.016	0.015	0.013	0.009	0.008	0.015	**0.733**[b]	0.013
BAN07p	0.001	0.013	0.015	0.013	0.015	0.013	0.003	0.014	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 other sites)*			
	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

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 documented 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. 2007; Forstner et al. 2008); toads were observed in Austin, Bastrop, Colorado, Lee, Leon, and Milam. Forstner et al. (2008) concluded that *B*. *houstonensis* is 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. 2007; 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, pers. obs.).

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 (pers. comm. to MRJF by Drs. Yantis and Dixon), and, unfortunately, no specimens exist for these counties. Additionally, the literature museum record searches found specimens from outside the known range (see **Appendix 1**): Brazos, Freestone (Yantis 1990), Houston, and Travis. These specimens have been requested and will 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. 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 more accurately resolve 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, Appendix # 2) are compared to the GLR p12 *B*. *houstonensis* (*n* = 11, four haplotypes, Appendix # 2), 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 10000 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 #). *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 per million years for the D-loop (Stöck et al. 2006), the

date of divergence for *B*. *americanus* and *B*. *woodhousii* is estimated at 2.283 to 0.457 million years ago (mya), and divergence between *B*. *americanus* and *B*. *houstonensis* is estimated at 2.627 to 0.342 mya. While the use of molecular clocks is contested at best (Maxson 1984; Hillis 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 often proposed date of 10000 years ago and likely existed during and possibly 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, there is evidence for hybridization among species (Fig. 2, Hillis & Price 1993; Masta et al. 2002; Pauly et al. 2004) and for reticulate relationships among populations of *B*. *houstonensis* (Fig. 4, Hillis & Price 1993). Evidence from both the phylogeny and the statistical parsimony network do not rebut *B*. *houstonensis* as a distinct lineage.

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

	B. cognatus	*B. americanus*	*B. fowleri*	*B. houstonensis*	*B. woodhousii*
B. cognatus	0.227	9.612 – 8.819	11.098 – 10.202	9.498 – 9.276	9.507 – 9.506
B. americanus	15.368%	1.712 – 0.342	4.245 – 3.887	2.627 – 0.342	2.283 – 0.457
B. fowleri	17.509%	6.528%	3.096	4.592 – 3.772	4.347 – 3.784
B. houstonensis	15.585%	2.666%	6.827%	1.598 – 0.114	3.083 – 0.114
B. woodhousii	15.478%	3.368%	6.723%	3.822%	1.143 – 0.114

*mya = million years ago

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 softwares 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 since all individuals from Austin, Colorado, and Leon counties had some level of 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). This locus was most successful in 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 and 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}s among the nine groups indicate high levels of differentiation among groups >85 km apart (F_{ST}s >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}s = 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}s with increasing geographic distance but some high F_{ST}s 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}s 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 since 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). *B. houstonensis* has been shown to utilize a 5 m corridor adjacent to and along 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 (Forstner & Swannack 2004; Swannack et al. 2006) have shown that typical adults likely remain within 200 m of their breeding pond during the post breeding season (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). In this 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 was 500 m (Price 1992) and in 4 weeks was ~2000 m (Forstner et al. 2003; Price 2003). Fewer female accounts exist, but the longest distance recorded was 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 is only 132 m. In this 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, 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 population units

In addition to the genetic clustering findings, other lines of evidence were identified that support the hypothesis that there are nine discernable populations of *B*. *houstonensis* (BAPp, BAS06p, COLs, LEOp, MILs, N, S_1, S_2, and U). The 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}s = 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}s 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}s were found in Austin County (0.196-0.400, average 0.273, Table 17) indicating that this population is the most distinct. 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; USFWS 1984; Quinn et al. 1987). *B. 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 this study) in Colorado County. Over five years, ~400000 eggs, ~7000 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 at 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). Since 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 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 this 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 since *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 reported 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 a 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 adult toads are believed to be alive throughout the range (Michael R. J. Forstner, personal communication 2009). Effective population sizes are almost always smaller than census population sizes in anurans (Easteal 1985; Dodd Jr. & 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 since 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 doing so may have higher mortality through predation (USFWS 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 (USFWS 1984). Extirpation in Harris and Ft. Bend counties has been charged to both habitat change over many decades and severe drought during the 1950s (USFWS 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. 2007). Habitat fragmentation or alteration (including urbanization and conversion to pasture or agriculture) remains the chief direct threat (USFWS 1984, 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; USFWS 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 represents the corridors through which unidirectional juvenile or adult movement takes place. Drainages are the most likely corridor route for juveniles or adults, since 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 most areas 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). We have begun an explicit investigation of hybridization in *B*. *houstonensis*, and that study will be complementary to this population genetic assessment.

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. 2007); samples (most were samples used in this 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 in most of the areas that local extinctions are very probable, and if managers do not know that local extinctions have occurred, then conservation strategies will be ineffective. Even today, we do not understand if the low numbers of individuals detected outside of Bastrop County represent low recent numbers correspondent to the declines in Bastrop County over the period of this study, or if they are stable indicators of low overall populations in the other counties. We know that Lee County went from hundreds of toads in chorus in the early part of this decade to none during the most recent years, reinforcing the need for active monitoring and stewardship efforts outside of Bastrop County.

Secondly, increasing the numbers of toads in the wild must be achieved. As population sizes have fallen the remaining populations are only more likely to be affected by compounding impacts (e.g. allee effects) (Gaston, Fujii, and Forstner in review). The seemingly viable mechanism to reverse the declines is the improvement of juvenile survivorship. Estimates of juvenile survival in the wild are between 0.0075 and 0.015 (Greuter 2004; Swannack et al. 2009), and estimates of survival of juveniles to adulthood

are only 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 re-introductions 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 re-introductions 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. 2007). 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%, or 25 times the upper estimates for this value in the wild (Forstner et al. 2007). 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 life zones (ie. breeding, occupied, and dispersal) is more critical today than it has ever been. Habitat removal, disturbance, and degradation occurs

nearly continuously across occupied habitat without the required permitting and we are unaware of a single instance of negative consequences to the people or businesses responsible for these actions. The continued unpermitted direct take of individuals and habitat will result in the extinction of the species unless a dramatic change in the enforcement of the ESA occurs. While dispersal routes and distances have not been directly measured, substantial evidence for population connectivity within 4 km does exist (data this study). Taken as a direct measure of Houston toad dispersal, this would provide a required 4 km buffer for a given breeding site in order to account for dispersal in a meaningful way. Thus, in addition to breeding habitat and occupied habitat directly around a pond, corridors for dispersal between breeding sites must be protected. Given that numbers of females are so low and that females probably roam more than males, this is another reason that habitat quality and connectivity are even more critical now that populations across the range have declined to their current levels.

Chytrid fungus is often "accepted" to be of much less consequence to North American amphibians than to those in the tropics. Yet, it may be that the pathogen is or has affected Houston toad populations. The fungus is now documented from Bastrop County Houston toads and efforts should be made to directly ascertain the pathology of the strains observed on Houston toads. If it proves a virulent pathogen then it may be serious enough that all females observed should be briefly held in captivity, tested for chytrid fungus, and treated if necessary before being released. All females need to be healthy and breeding to stabilize or improve population sizes; further, infected females may be chytrid vectors because they move across the landscape. It follows that how chytridiomycosis affects *B. houstonensis* needs to be determined.

Ultimately, conservation managers must actively engaged the public in all of the remaining occupied counties, 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* occurs primarily on private lands, and similar outreach programs should be attempted as quickly as possible before further extirpations have occurred.

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Appendix 1. Voucher specimen records of *Bufo houstonensis**

County	Year	Institution	Institution ID	Collector	Locality	Prep	Reference
Austin			**9 specimens**		near Sealy		{Sanders, 1953 #1110}
Austin	1990			JH Yantis	~8 km E of New Ulm on FM1094, N of roadway, upper end of Swenson Lake	**sound recording only?**	{Yantis, 1990 #1221}{Price, 1990 #1121}
Bastrop	1951	TCWC	80731	R Osborne	2.5 mi NE Bastrop, off Hwy 21	ethanol	
Bastrop	1963	TCWC	70807-70817, 70836-70838, 70840-70864	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
Bastrop	1963	TCWC	70865, 70870	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
Bastrop	1963	TCWC	70869	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
Bastrop	1966	TNHC	50117	L Brown	BSP, 2 mi SE in Barking Dog Pond		
Bastrop	1966	TNHC	62425			skeleton	
Bastrop	1967	MVZ	81946-81947	RD Sage	BSP	ethanol	
Bastrop	1968	TCWC	82097	H Quinn	4 mi N, 1 mi W Bastrop, BSP entrance off Hwy 21	skeleton	
Bastrop	1970s	TCWC	80729-80730	HZG	2.5 mi NE Bastrop, off Hwy 21	ethanol	
Bastrop	1971	MVZ	99123-99125	SS Sweet	0.5 mi SE BSP	ethanol	
Bastrop	1978	TCWC	70542, 70544-70560, 70566-70598, 70604, 70607,	HZG	6.44 km N BSP entrance, 1.61 km W	larval and embryo	

			70609-70611, 70614, 70623-70631				
Bastrop	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	
Bastrop	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	
Bastrop	1979	TCWC	70634-70647, 70649-70704	HZG	5.47 km N BSP entrance, Hwy 21, 2.4 km E	ethanol	
Bastrop	1979	TCWC	72908	HZG	5.49 km N BSP, 2.4 km E Hwy 21	ethanol	
Bastrop	1979	TNHC	50622	D Hillis, D Mosier	in pond 2.6 mi from BSP entrance on Hwy 21		
Bastrop	1980	KU	190153-190154		no data	ethanol	{Thomas, 1982 #1518}
Bastrop	1980	TCWC	60035-60036			ethanol	{Thomas, 1982 #1518}
Bastrop	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	
Bastrop	1980	TCWC	70712-70725, 70728-70732, 70734	HZG	3.3 mi N BSP entrance, Hwy 21, 1 mi E, permanent pond	larval and embryo	
Bastrop	1980	TCWC	70818-	HZG	3.3 mi N	ethanol	

			70825, 70828-70834		BSP entrance, Hwy 21 (Jim Small Ranch), 1 mi E (across from power line)		
Bastrop	1981	TCWC	70785-70786	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
Bastrop	1981	TNHC	49375-49379	D Hillis	BSP entrance, 5 km NE along Hwy 21; collected at site 4, at roadside pond: 'Big Fence'		{Hillis, 1984 #1054}
Bastrop	1981	TNHC	49380	D Hillis	BSP entrance, 7 km NE along Hwy 21; collected at site 3, at roadside pond: 'Trailer Pit Pond'		{Hillis, 1984 #1054}
Bastrop	1981	TNHC	49381-49383	D Hillis	BSP entrance, 4 km NE along Hwy 21; collected at site 5, at roadside pond: '4 Score Pond'		{Hillis, 1984 #1054}
Bastrop	1981	TNHC	49384-49392	D Hillis	BSP entrance, 2 km NE along Hwy 21; collected at site 6, at roadside pond: 'Bog Pond'		{Hillis, 1984 #1054}
Bastrop	1981	TNHC	49394	D Hillis	BSP entrance, 4 km NE along Hwy 21; collected at site 5, at roadside		{Hillis, 1984 #1054}

					pond: '4 Score Pond'		
Bastrop	1981	TNHC	49395	D Hillis	FM 1441, 4 km NW jct Hwy 21; collected at site 2, at roadside pond: 'Twin Ponds'		{Hillis, 1984 #1054}
Bastrop	1982	TCWC	60605	JR Dixon	2.5 mi W Hwy 21, Hwy 1441	ethanol	
Bastrop	1982	TCWC	60679, 60683	JR Dixon & HK McCrystal	0.6 mi NW Hwy 21, Hwy 1441	ethanol	
Bastrop	1982	TCWC	60680	JR Dixon & HK McCrystal	BSP	ethanol	
Bastrop	1982	TCWC	60681	JR Dixon & HK McCrystal	2.4 mi NW Hwy 21, Hwy 1441	ethanol	
Bastrop	1982	TCWC	60682	JR Dixon & HK McCrystal	2.2 mi N Bastrop	ethanol	
Bastrop	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	
Bastrop	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	
Bastrop	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	
Bastrop	1983	TCWC	82098	H Quinn	4 mi N, 1 mi W Bastrop, BSP entrance off Hwy 21	skeleton	
Bastrop	1987	TCWC	70756	HZG	no data	ethanol	

Bastrop	1988	TCWC	70757, 70774, 70777	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
Bastrop	1988	TCWC	70763, 70772, 70775	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
Bastrop	1988	TCWC	70765	HZG	3.6 mi N Hwy 21, Chapman Ranch, Houston Zoo 1980 study site B.	ethanol	
Bastrop	1988	TCWC	70767	HZG	3.3 mi N entrance to BSP, Hwy 21, 0.75 mi SE Hidden Lake (Jim Small Ranch)	ethanol	
Bastrop	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	
Bastrop	1988	TCWC	70771, 70781-70782, 70788, 90302	HZG	no data	ethanol	
Bastrop	1988	TNHC	64553-64554	Hillis & Cocroft	TX rte 21, 0.8 km SW jct FM	alcohol	
Bastrop	1989	TCWC	67799-67800	JR Dixon	BSP, 2 mi S Bastrop	ethanol	
Bastrop	1989	TCWC	70737-70739	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
Bastrop	1989	TCWC	70798-70800	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
Bastrop	1990	BLB	17445	RH Benson	BSP	sound recording	
Bastrop	1990	TCWC	67563-67566	AH Price	BSP, study pond #10	ethanol	{Price, 1990 #1121}
Bastrop	1990	TCWC	67898	MJ Whiting	Hwy 21, 1.3 mi, plus 29	ethanol	{Price, 1990 #1122}

					yards E BSP, S side		
Bastrop	1990	TCWC	68210	MJ Whiting	Hwy 21 (S side), 4.4 mi plus 15 yds E BSP	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	68214	MJ Whiting	Hwy 21, 3.85 mi E BSP, N side	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	68255, 68258	MJ Whiting	Hwy 21, 1.3 mi plus 20 yds E BSP, S side	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	68256	MJ Whiting	Hwy 21 (S side), 4.6 mi E BSP	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	68257	MJ Whiting	Hwy 21, 0.6 mi, 23 yards E BSP, S side	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	68259	MJ Whiting	Hwy 21, 2.1 mi E BSP, N side	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	69812	RC Murray	4.1 mi - 60 yds E BSP entrance on S side of Hwy 21	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	69813	RC Murray	4.3 mi E BSP entrance on N side of Hwy 21	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	69815	RC Murray	1.3 mi E BSP entrance on N side Hwy 21	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	69816	RC Murray	3.1 mi - 38 yds E BSP entrance, S Hwy 21	ethanol	{Price, 1990 #1122}
Bastrop	1990	TCWC	70804-70805	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
Bastrop	1991	TCWC	70759	HZG	3.6 mi N Hwy 21, Chapman Ranch, Houston Zoo 1980 study site B.	ethanol	
Bastrop	1991	TCWC	70803	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
Bastrop	1991	TNHC	55492-	AH Price	BSP, pond #5	alcohol	{Price, 1992

			55516				#1123}
Bastrop	1992	TCWC	82099	H Quinn	4 mi N, 1 mi W Bastrop, BSP entrance off Hwy 21	skeleton	
Bastrop	1993	TCWC	70376	Texas Dept. of Public Highways	Hwy 21 1.5 mi E FM 1441	ethanol	
Bastrop	1993	TCWC	71657	JR Dixon	0.3 mi S Hwy 71 on Co Rd 191, then 1 mi E on gravel road	ethanol	
Bastrop	2003	TCWC	87316-87317	MRJ Forstner		ethanol	
Bastrop	2004	TCWC	90257-90261	MRJ Forstner	GLR		
Bastrop	2004	TCWC	90753-90754	T Swannack	GLR		
Bastrop	2005	TCWC	90736	M Jones	BSP, burn area		
Bastrop		TCWC	87318	MRJ Forstner		ethanol	
Bastrop		TCWC	90751-90752	T Swannack	GLR		
Bastrop		TCWC	70741, 70758	HZG	4.0 mi N BSP entrance, Hwy 21, 1.0 mi W	ethanol	
Bastrop		TCWC	70742	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
Bastrop		TCWC	70743	HZG	3.3 mi N entrance to BSP, Hwy 21, 0.75 mi SE Hidden Lake (Jim Small Ranch)	ethanol	
Bastrop		TCWC	70747, 70749, 70755	HZG	6.44 km N BSP entrance, 1.61 km W	ethanol	
Bastrop		TCWC	70791, 70794	HZG	1.2 mi N BSP entrance, Hwy 21, pond E Hwy, 100 m off rd	ethanol	
Bastrop		TCWC	70795-	HZG	6.44 km N	ethanol	

			70797, 70801-70802, 70806		BSP entrance, 1.61 km W		
Bastrop		TCWC	90303-90304	HZG			
Bastrop		TNHC	34740, 35536-35537		Bastrop		{Thomas, 1984 #1354}
Brazos	1962	MSUM	HE.8877	EM Schwille	4.2 mi. NE of Peach Creek community, along dirt road	fluid	
Burleson	1950	TCWC	7068-7069	JL Robertson	4 mi N Caldwell	ethanol	{Sanders, 1953 #1110}
Burleson	1989			JH Yantis	3.3 mi SW of Caldwell via TX Hwy 21 to jct with RR908, then ~2 mi N to Cade Lakes		{Price, 1990 #1121}
Colorado		UMMZ	127826		6 mi E Columbus		{Sanders, 1953 #1110}
Colorado			**8 specimens**		6 mi E Columbus		{Sanders, 1953 #1110}
Colorado	1982	TCWC	62388	K King	Attwater Prairie Chicken National Wildlife Refuge	ethanol	
Colorado	1990			JH Yantis	~9 km S of New Ulm via county rds, 200 m E of county rd & just N of Hayes Creek	**sound recording only?**	{Yantis, 1990 #1221}{Price, 1990 #1121}
Colorado	1990			JH 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 only?**	{Yantis, 1990 #1221}{Price, 1990 #1121}
Freestone	1990			JH Yantis	~8 km S & 5 km E of Lanely by county rds, E side of	**to TMM after tissue 4allozyme**	{Yantis, 1990 #1221}{Price, 1990 #1121}

					county rd & E side of triangle driveway		
Harris	1950s	TCWC	80724-80728	Wottring	Houston	ethanol	
Harris	1950	CM	29172	Wottring	Fairbanks	alcohol	
Harris	1950	CU	5538	Wottring	Houston	fluid	
Harris	1950	TNHC	28860	Wottring			
Harris	1951	CU	5499	Wottring	NW of Houston, Fairbanks	fluid	
Harris	1951	USNM	542212	AP Blair	1 mi S of Houston airport	ethanol	
Harris	1952		**10 skeletons**	Wottring & WJ Greer	Fairbanks	skeleton	{Sanders, 1953 #1110}
Harris	1952	CAS	12768-12769*	Wottring & WJ Greer	Fairbanks		{Sanders, 1953 #1110}
Harris	1952	CM	32688-32690*	Wottring & WJ Greer	Fairbanks	alcohol	{Sanders, 1953 #1110}
Harris	1952	MCZ	A-28019-A-28022*	Wottring & WJ Greer	Fairbanks	alcohol	{Sanders, 1953 #1110}
Harris	1952	SDNHM	42043-42044, 42049-42050		no data		
Harris	1952	UCM	11924	Wottring			
Harris	1952	UIMNH	33687-33689*	Wottring & WJ Greer	Fairbanks		{Sanders, 1953 #1110}
Harris	1952	UMMZ	127825	Wottring & WJ Greer	Fairbanks		{Sanders, 1953 #1110}
Harris	1952	UMMZ	127827	Wottring			
Harris	1952	USNM	134433-134436*	Wottring & WJ Greer	Fairbanks	ethanol	{Sanders, 1953 #1110}
Harris	1952	USNM	542211	AP Blair	Fairbanks	ethanol	
Harris	1958	TNHC	25626-25630	Wottring	Houston		{Thomas, 1984 #1354}
Harris	1959	CM	63376	Wottring	Houston	alcohol	
Harris	1988	TCWC	70776	HZG	HZG	ethanol	
Harris	1989	TCWC	70539-70540	HZG	HZG	larval and embryo	
Harris	1989	TCWC	70541	HZG	HZG	ethanol	
Harris		FLMNH	12948	Mr. Tabony			
Harris		FMNH	74725	Wottring			
Harris		LACM	87721	D Hansaker, Giht, &	Houston		

				WF Blair			
Harris		**SM or BCB?**	**5441, 5457-5459, 5461-5462, 5678-5679, 5683, 5690-5691, 5693**				{Thomas, 1984 #1354}
Harris		TCWC	70746, 70760-70761	HZG	HZG	ethanol	
Harris		TNHC	34741		Houston		{Thomas, 1984 #1354}
Houston	1959	LSU	9307-9313	Fox & Wottring	no data	isopropanol	
Houston	1970	TTU	5080-5082	V Wade	Ratcliff Lake Reservoir	alcohol	
Lavaca	1991	TNHC	56005	R Lehman	Hallettsville, SE at Upper Laughlins Sandy Creek in pond	alcohol	{Yantis, 1991 #1219}
Lee	2001	TCWC	84556	JR Dixon	0.3 mi E Bastrop/Lee Co. line on County rd 333	ethanol	{Gaston, 2001 #1128}
Leon	1989			JH Yantis	pond 50-100 m W of Cherokee Lake	**to TMM**	{Yantis, 1989 #1218}{Price, 1990 #1121}
Leon	1989		**2 specimens**	JH Yantis	water filled depression ~10 m E of Cherokee Lake	**to TMM**	{Yantis, 1989 #1218}{Price, 1990 #1121}
Leon	1989			JH Yantis	water filled depression in RV park	**to TMM**	{Yantis, 1989 #1218}
Leon	1990	TCWC	68265-68270	JH Yantis	Hilltop Lake	ethanol	
Leon	1990		**3 specimens**	JH Yantis	trailer park E of Cherokee Lake	**to TMM after tissue 4allozyme**	{Price, 1990 #1121}
Leon	1991	TNHC	55580-55590	JH Yantis	Hilltop Lakes Estates, vicinity of Cherokee Lake	alcohol	
Liberty	1950s			W Gottsch	6 mi S Liberty		{Sanders, 1953 #1110}
Milam	1987	TCWC	65498		6.2 mi SW Rockdale	ethanol	

Robertson	1975	TCWC	53989-53990	OW Thornton Jr	10.0 mi WSW of Wheelock, EG Marsh farm	ethanol	
Robertson	1989			JH Yantis	water filled sand pit, ~6 mi E Hearne on TX Hwy 391 then just N of Hwy on two-track	**to TMM after tissue 4allozyme**	{Yantis, 1989 #1218}{Price, 1990 #1121}
Robertson	2000	TCWC	84246	TJ Hibbitts	5.6 mi W Jct FM 46/391 on 391	ethanol	
Travis	1952	INHS	6373	PW Smith & SA Minton			
Houston?	1969	LSU	47849		Sam Houston State Park	isopropanol	
unknown	1961	TNHC	62426			skeleton	
unknown		TNHC	60459, 60722			alcohol	
unknown		TNHC	60879			skeleton	

BSP = Bastrop State Park , GLR = Griffith League Ranch, HZG = Houston Zoological Gardens, * 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, Toby J. Hibbitts, and Travis J. Laduc, Greg Schneider, **Carl Franklin**: Texas Natural History Collection, Texas Memorial Museum, Austin; Texas Cooperative Wildlife Collection, College Station; Museum of Zoology, University of Michigan, Ann Arbor; and **UTA (University of Texas at Arlington, Arlington**).