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PHYLOGENETIC AND POPULATION GENETIC ASSESSMENT OF RAFINESQUE'S BIG-EARED BAT *(CORYNORHINUS RAFINESQUII)*

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Abstract—Rafinesque's big-eared bat (*Corynorhinus rafinesquii*) is distributed across the Southeastern United States. Due to habitat loss and low population numbers, this species is a Federal species of concern and protected by every State within its range. Effective management of any species of concern is dependent on an unambiguous understanding of taxonomic relationships. However, for this species, there are discordant inferences about subspecific designations from previous studies. Further, there have been no assessments of population genetic status for this species. Such assessments could provide information on genetic diversity and population connectivity and increase our understanding of the need for management and conservation of this species. Therefore, our goals were to assess population level genetic diversity and connectivity among 5 colonies in Arkansas (139 individuals) and to infer the evolutionary relationships of these bats to *C. rafinesquii* collected across its distribution (additional 216 individuals). We used mitochondrial DNA control region sequences and 11 microsatellite loci to infer genetic relationships, estimate levels of genetic diversity, and examine population connectivity among 5 colonies in Arkansas. Although we identified two phylogenetically divergent mitochondrial DNA lineages, these correspond to neither current subspecific designation nor nonoverlapping geographical groups. Genetic diversity and population connectivity estimated from mitochondrial DNA was high in Arkansas populations probably due to occurrence of both evolutionary lineages within each colony. However, estimates from microsatellite DNA of genetic diversity, population connectivity, and effective population sizes in these populations were low. Further, our results suggested a weak signal of population bottleneck in Arkansas colonies and low genetic connectivity. Current conservation efforts should continue to focus on protection of roosts and improvement of habitat corridors to connect populations.

INTRODUCTION

Rafinesque's big-eared bat (*Corynorhinus rafinesquii*) is a medium-sized bat that ranges across a broad portion of the Southeastern United States (fig. 1; Bayless and others 2011). However, there has been concern about its status since the mid-20th century due to low population numbers and patchy distribution. Handley (1959) expressed concern for the status of this species based on lack of known large colonies and limited numbers of museum specimens. He concluded that populations had declined due to anthropogenic impacts in parts of their range. Jones and Suttkus (1975) published data from a 9-year study in Louisiana and concluded that these bats were rare likely due to severe population declines. They also concluded that reduction occurred because *C. rafinesquii* is sensitive to disturbance from humans at vulnerable maternity roost sites which were principally found in abandoned manmade structures. Based on concerns over status of this species, it was listed as vulnerable to extinction on the 2004 International Union for Conservation of Nature Red List, a Federal species at risk (U.S. Fish and Wildlife Service 1985), and a species of concern in every State, except Virginia, where they are considered endangered (Bayless and others 2011, Kentucky State Nature Preserves Commission 1996, Lance 1999).

In spite of conservation concerns, little is known about evolutionary relationships, genetic diversity, and gene flow among populations of *C. rafinesquii*. For any species of concern, it is important that evolutionary relationships, or taxonomy, of that species is understood. In a taxonomic revision of the genus *Corynorhinus* based on morphological characters, Handley (1959) designated two subspecies of *C. rafinesquii* assigning populations from the Southeastern United States (Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, eastern Oklahoma, South Carolina, eastern Texas, and coastal Virginia) to *C. r. macrotis* and populations from East Central United States (northern Alabama, Arkansas, northern Georgia, Kentucky, southern Illinois, southern Indiana, northern Mississippi, eastern Missouri, western North Carolina, southern Ohio, eastern Oklahoma, western South Carolina, Tennessee, western Virginia, western West Virginia) to *C. r. rafinesquii*, with areas where the two subspecies overlapped (Handley 1959:152). More recently, Piaggio and Perkins (2005) tried to elucidate evolutionary relationships of *C. rafinesquii* using
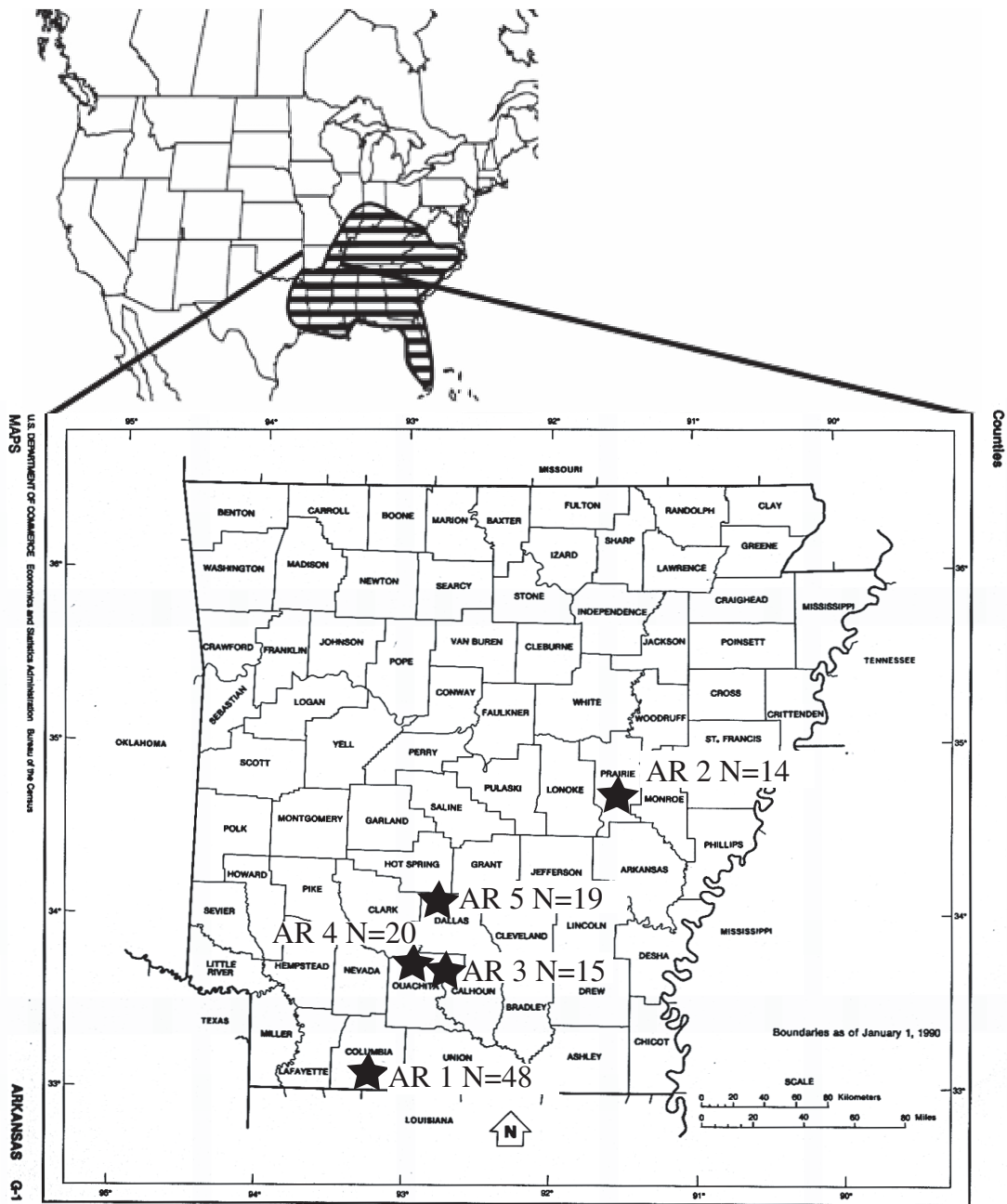
Figure 1—Distribution of *Corynorhinus rafinesquii* with Arkansas roosts sampled shown in detail (AR1, AR2, AR3, AR4, and AR5) and number of individuals sampled per colony noted. (Map from the University of Texas, Austin, TX, Perry Casteñeda Library map collection online http://www.lib.utexas.edu/maps/.)

limited sampling from portions of the species' range and both mitochondrial and nuclear DNA sequences. That study found that there was no correlation between the two designated subspecies of *C. rafinesquii*, from Handley (1959), and the molecular phylogeny. Piaggio and Perkins (2005) concluded that a more detailed study of *C. rafinesquii* including more samples representing a greater portion of their range was required to determine if any subspecific designation was warranted. To correctly determine conservation status of this species, it is critical that evolutionary relationships, and, thus,

taxonomy and geographical boundaries of taxonomic units, are understood. A molecular phylogenetic approach such as the one used in Piaggio and Perkins (2005) with additional samples from across the range of *C. rafinesquii* could provide such information.

Piaggio and Perkins (2005) found that there were two divergent evolutionary lineages of *C. rafinesquii*. However, both clades had samples from Arkansas and some samples in each clade were from the same colonies. After more than 10

years of study, there have been only five known continually active colonies of *C. rafinesquii* in Arkansas, all of which occupy abandoned humanmade structures (Saugey 2000). These colonies are found in a region of Arkansas that was historically dominated by bottomland hardwood forests which have been largely converted to agricultural uses and are one of the most endangered forest types in the United States (Abernathy and Turner 1987, The Nature Conservancy 1992, Turner and others 1981). It is assumed that mature, hollow trees in the bottomland hardwood forests represent historical roosting habitat for *C. rafinesquii* (Clark 1990, 1991). Therefore, it appears that these bats may use abandoned manmade structures because of loss of natural roosts. Because all of the known *C. rafinesquii* colonies in Arkansas were located in abandoned manmade structures, there was concern that these colonies were remnant populations and that they may have lost connectivity and suffered reduced genetic diversity due to the loss of contiguous bottomland habitat. Further, these colonies were considered threatened due to the ephemeral nature of their roosts.

Maintenance of genetic diversity within populations and connectivity among genetically diverse populations is crucial for sustaining the evolutionary potential of a species (England and others 2003). A loss of population connectivity as a result of reduced and/or fragmented habitat may increase susceptibility to a population bottleneck (Cornuet and Luikart 1996), which can allow genetic drift to affect a population resulting in low effective population size, loss of genetic diversity, and inbreeding. Such populations are likely more susceptible to disease, ecological catastrophes, and eventual extinction, thus, impacting evolutionary potential of that species (Altizer and others 2003, Lacy 1997). Analyses of maternally inherited mitochondrial DNA (mtDNA) and biparentally inherited autosomal microsatellites can be used to infer genetic relationships and to estimate various population parameters including genetic diversity, population connectivity, and effective population sizes (Avise 1995, Avise and Hamrick 1996, Haig 1998). If populations exhibit genetic evidence of population bottlenecks, reduced genetic diversity, and/or reduced effective population sizes, then targeted conservation efforts and management practices are needed.

Given the conservation status of *C. rafinesquii* and lack of data regarding genetic diversity for this species, we employed genetic markers, both mtDNA and microsatellites, to infer evolutionary relationships of *C. rafinesquii* with samples from across its range and to estimate genetic diversity, connectivity among populations, and effective population sizes among Arkansas colonies. We predicted that due to past habitat loss and subsequent disjunction and/or population reduction, we would detect population bottlenecks. If true, estimates of genetic diversity and population connectivity would be low and there might also be inbreeding and low effective population sizes. This, in turn, would guide recommendations for species' conservation from a genetic perspective.

MATERIALS AND METHODS

Study Area

We collected samples from across the Southeastern United States. The study area in Arkansas included widely spaced locations in Columbia, Dallas, and Ouachita Counties within the Tertiary Uplands of the southcentral Plains and Prairie County within the Grand Prairie of the Mississippi River Alluvial Plain, Arkansas (Arkansas Natural Heritage Commission 2003). The Tertiary Upland sites were dominated by commercial shortleaf and loblolly pine (*Pinus echinata* and *P. taeda*) plantations that largely replaced native oak-hickory-pine (*Quercus* spp.-*Carya* spp.) forests except in narrow streamside zones (Woods and others 2004). Forested tracts were interspersed with bayous and by pasture for grazing cattle. Most of the large bottomland hardwood timber had been harvested (Dahl 1990). The Grand Prairie was a loess-covered terrace that once contained an extensive tall grass prairie converted to cropland in the early 20th century (Holder 1970). Average precipitation was 127 cm, and average temperatures are highest in July (average 32 °C) and lowest in January (7 °C). Expansive areas of rice, soybeans, cotton, corn, and wheat were cultivated in the area (Woods and others 2004). Braided bayous were found throughout this area with bottomland hardwood forests occurring along drainages and floodplains, upland hardwood forests along hills and bluffs, and hardwood savannas along the edges of prairie terraces. Forested acres had been reduced by more than half through conversion to croplands and development (Shepherd 1984). The eastern border of the Grand Prairie was adjacent to the White River riparian area that contained some of the most extensive areas of remaining bottomland hardwood forests in Arkansas (Woods and others 2004).

Sample Collection

We collected tissue samples during 2000 to 2005 at five roosts in Arkansas (fig. 1). Sites AR1 (48 individuals), AR2 (14 individuals), and AR5 (33 individuals) were maternity roosts; AR3 (15 individuals) and AR4 (29 individuals) were hibernacula. However, the hibernacula used by individuals from AR1 and AR5 and the maternity roosts for individuals from AR3 and AR4 were known and adjacent to sites where samples were collected, e.g., abandoned house used as a maternity roost and adjacent well used as a hibernaculum. Therefore, we assumed that each of these sites represented a single and separate colony. Further, based on approximately 12 years of mark-recapture data representing 3,500 captures of bats at these sites, exchange of individuals was uncommon among sampled sites even when they were proximate (< 14 km) to one another. Therefore, we assumed that each sampled maternity roost or hibernation site was a single colony. We also sequenced DNA from 216 other individuals from other parts of the range of *C. rafinesquii* (Florida, Kentucky, Mississippi, North Carolina, Tennessee,

South Carolina, Louisiana, and Texas) and 5 other Arkansas individuals, and we included these in our phylogenetic analysis (table 1; fig. 1).

We captured individual *C. rafinesquii* in Arkansas colonies found in wells using a method employing an umbrella (England and Saugey 1999). When bats were found in abandoned buildings, we used hand nets for capture. Bats collected outside of Arkansas were captured using mist nets. We collected a 3-mm tissue biopsy from the right wing (Worthington Wilmer and Barratt 1996) before releasing bats at site of capture. Capture and sampling protocols were reviewed and approved by the University of Colorado, Boulder's Institutional Animal Care and Use Committee. We preserved samples in a 20-percent dimethyl sulfoxide and a 0.25M-ethylenediaminetetraacetic acid solution saturated with sodium chloride and optimized at pH 8.0 (Seutin and others

1991). We used half of each wing punch to extract genomic DNA using a DNeasy Blood & Tissue Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's protocol.

DNA Amplification, Sequencing, and Genotyping

We amplified genomic DNA from each sample and the mtDNA control region was sequenced, following protocols described in Piaggio and Perkins (2005). We genotyped *C. rafinesquii* from Arkansas roosts (AR1, 48 individuals; AR2, 11 individuals; AR3, 15 individuals; AR4, 20 individuals; and AR5, 18 individuals) using 11 loci: EF15B, EF20C, EF21, EF14 (Vonhof and others 2002), NN8 (Petri and others 1997), PAUR 05 (Burland and others 1998), Cora_D12_D12, Cora_E07_E07, Cora_H07_C05, Cora_B07_H12, and Cora_E10_G03 (Piaggio

Table 1—Genetic samples of Rafinesque's big-eared bats (*Corynorhinus rafinesquii*) sequenced and analyzed with localities, ownership/donator, and GenBank accession numbers indicated

Taxon	Locality[a]	Donor/owner[b]	Pop[c]	Acc no[d]
C. mexicanus	Guacaceví, Durango, Mexico	CIIDIR CRD 3110 Celia López-González		AY713590
	Guanacveí, Durango, Mexico	CIIDIR CRD 3125 Celia López-González		AY713591
	Guanaceví, Durango, Mexico	CIIDIR CRD 3115 Celia López-González		AY713593
	Milpa Alta, Distrito Federal, Mexico	Rafael Avila-Flores		AY713785
C. rafinesquii	Arkansas, Columbia	USFS David Saugey	AR1	AY713635–AY713643 AY713666–AY713675 AY713684–AY713696 AY713717–AY713731
	Arkansas, Prairie	AGFC Blake Sasse	AR2	AY713652–AY713665
	Arkansas, Ouachita	USFS David Saugey	AR3	AY713900–AY713909 AY775995–AY775999
	Arkansas, Ouachita	USFS David Saugey	AR4	AY713910–AY713919 AY775976–AY775985 HQ239099–HQ239102 HQ239107–HQ239111
	Arkansas, Dallas	USFS David Saugey	AR5	AY713920–AY713929 AY775986–AY775994 HQ239095–HQ239098 HQ239112–HQ239121
	Florida, Osceola	Laura Finn Kelli Deichmueller		AY713789–AY713790
	Florida, Holmes	FFWCC Jeff Gore		AY713818
	Kentucky, Estill	KDFWR Traci Wethington		AY713877–AY713878 AY713881–AY713882
	Kentucky, Bath	Eric Britzke		AY713786–AY713788
	Louisiana, Union Parish	Chris Rice		HQ239178–HQ239194

continued

Table 1—Genetic samples of Rafinesque's big-eared bats (*Corynorhinus rafinesquii*) sequenced and analyzed with localities, ownership/donator, and GenBank accession numbers indicated (continued)

Taxon	Locality[a]	Donor/owner[b]	Pop[c]	Acc no[d]
C. rafinesquii (continued)	Mississippi, Perry	Austin Trousdale		AY713842–AY713854
	Mississippi, Wayne	Austin Trousdale		AY713855–AY713860
	Mississippi, Jones	Austin Trousdale		AY713861
	Mississippi, Noxubee	USFWS David Richardson		HQ239077–HQ239092
	North Carolina, Bladen	Mary Kay Clark		AY713595–AY713620
	South Carolina, Charleston	Heather Thomas		AY713698–AY713701 AY713751–AY713756 HQ239093–HQ239094 HQ239103–HQ239106
	South Carolina, Oconee	SCDNR Mary Bunch		AY713767
	South Carolina, Pickens	SCDNR Mary Brunch		AY713768
	South Carolina, Richland	SCDNR Mary Bunch		AY713792
	South Carolina, Dorchester	Piaggio		AY713791
	South Carolina, Orangeburg	Frances Bennett		AY713819–AY713820 AY713822
	South Carolina, Kershaw	Frances Bennett		AY713821
	South Carolina, Barnwell	Frances Bennett		AY713823
	South Carolina, Williamsburg	Frances Bennett		AY713824–AY713825 AY713827–AY713828
	South Carolina, Georgetown	Frances Bennett		AY713826
	South Carolina, Colleton	Frances Bennett		AY713829–AY713830
	Tennessee, Chester	Brian Carver		HQ239122–HQ239152
	Tennessee, Fentress	Mary Kay Clark		HQ239153–HQ239177
	Texas, Harrison	Leigh Stuemke/Chris Comer		HQ239208–HQ239209 HQ239213–HQ239215 HQ239218–HQ239220 HQ239223–HQ239225 HQ239228–HQ239230
	Texas, Liberty	Leigh Stuemke/Chris Comer		HQ239199 HQ239203–HQ239207 HQ239210–HQ239212 HQ239216–HQ239217 HQ23922–HQ239222 HQ239226–HQ239227 HQ239231–HQ239232
	Texas, Polk	Leigh Stuemke/Chris Comer		HQ239195–HQ239198 HQ239200–HQ239202

CIIDIR = Colección Regional Durango (Vertebrados), CIIDIR Durango, Instituto Politécnico Nacional, México; USFS = U.S. Forest Service, Ouachita National Forest; AGFC = Arkansas Game and Fish Commission; FFWCC = Florida Fish and Wildlife Conservation Commission; KDFWR = Kentucky Department of Fish and Wildlife Resources; USFWS = U.S. Fish and Wildlife Service, Noxubee National Wildlife Refuge; SCDNR = South Carolina Division of Natural Resources.

[a] State, county (or city, State, country).

[b] Person and/or organization that donated tissue and/or owns sample; museum catalog numbers provided when possible.

[c] Population belongs to; applicable only to Arkansas populations surveyed in detail in this study.

[d] GenBank accession number.

and others 2009a). We amplified products from these loci via polymerase chain reaction (PCR) with one primer end-labeled with TET, FAM, or HEX fluorescent label (Sigma-Genosys Co., USA). We amplified each microsatellite PCR for the primers designed from other bat species in a standard 25 μl reaction which contained optimized amounts of PCR water; 5X buffer C (Invitrogen by Life Technologies Corp., USA); 2.5 μl of dNTP (10 mM; Invitrogen by Life Technologies Corp., USA); 2.5 μl of each primer (1 pM/μl); Taq DNA polymerase (Promega Corp., USA); and 1 μl of genomic DNA. Amplification consisted of an initial denaturation at 94 °C for 2 minutes followed by 30 cycles of denaturing at 94 °C for 30 seconds, annealing at 56 °C (PAUR05 and EF15), 52 °C (EF21), or 46 °C (EF14, EF20C, and NN8) for 45 seconds, and extension at 72 °C for 45 seconds with a final extension period of 7 minutes at 72 °C. Amplification protocols for the *C. rafinesquii* primers are described in Piaggio and others (2009a).

We visualized genotypes from the primers designed from other bat species and some sequencing products on acrylamide gels on a MJ BaseStation 51™ sequencer (MJ Bioworks, Inc., Sauk City, WI). We scored microsatellite alleles with Cartographer 1.2.6 software (MJ Bioworks, Inc., Sauk City, WI) and confirmed these by manual examination. We visualized the *C. rafinesquii* specific microsatellites and remaining sequences on an AB 3130 (Applied Biosystems by Life Technologies Corp., Foster City, CA) automated genetic analyzer and scored with ABI GeneMapper® Software.

Sequence Analyses

We generated alignments of mtDNA control region sequences using Sequencher® 4.9 (Gene Codes Corp., Ann Arbor, MI) and checked by eye. We used *C. mexicanus* sequences generated from a previous study (Piaggio and Perkins 2005) as an outgroup for phylogenetic analyses because this is the sister taxon to *C. rafinesquii* (Hoofer and Van Den Bussche 2001, Piaggio and Perkins 2005). We completed maximum likelihood phylogenetic analyses using RAxML (Stamatakis 2006, Stamatakis and others 2008) available through Web-based Cyberinfrastructure for Phylogenetic Research (CIPRES) supercomputer [http://www.phylo.org/. (Date accessed: November 19, 2010)]. We implemented the estimation of the general time reversible substitution model with gamma distributed rate variation estimation using RAxML (Stamatakis and others 2005). We evaluated bootstrap analysis of nodal support with number of pseudoreplicates automatically generated by the program. We visualized the maximum likelihood tree output and edited for publication and a radial tree layout of this tree was generated in FigTree v.1.2.1 [http://tree.bio.ed.ac.uk/software/figtree/. (Date accessed: November 19, 2010)].

We quantified genetic diversity from DNA sequence data as number of individuals sequenced per population, number of unique haplotypes, haplotype diversity, nucleotide diversity (Nei 1987), parsimony informative sites, and average pairwise differences within Arkansas populations and other populations where there was adequate sample size for comparison (Union Parish, LA, $n = 17$; Noxubee County, MS, $n = 16$; Blanden County, NC, $n = 26$; Chester County, TN, $n = 31$; Fentress County, TN, $n = 25$; Liberty County, TX, $n = 17$). To evaluate how genetic diversity was distributed among Arkansas populations, we first estimated population differentiation using F_{ST} (Weir and Cockerham 1984) and ascertained significant substructure between populations with 5,000 randomization tests. We used sequential Bonferroni corrections to compute critical significance levels for these data (Rice 1989). We then evaluated the relationship between population differentiation (Slatkin's linear $F_{ST}/(1 - F_{ST})$; Slatkin 1993) and log-transformed geographic distances ($\log_{10}$km) to determine if there was isolation-by-distance (IBD). We also used this method to test for IBD across the range of the species by using the Arkansas populations and other populations from across the range where adequate sample size was collected (see above). We appraised nested levels of variation among colonies and within colonies using an analysis of molecular variance (AMOVA; Excoffier and others 1992) with 9,000 permutations. We performed these evaluations using Arlequin ver. 3.1 (Excoffier and others 2005) except for the calculation of the parsimony informative sites, which we evaluated with PAUP* 4.0b (Swofford 2002).

Microsatellite Analyses

We assessed microsatellite loci for null alleles using Micro-Checker (Van Oosterhout and others 2004). We also tested loci for significant departures from Hardy-Weinberg equilibrium (HWE) with 9,000 steps of a Markov chain and significant evidence of linkage disequilibria among loci using Arlequin ver. 3.1 (Excoffier and others 2005). We used sequential Bonferroni corrections to compute critical significance levels for multiple tests using these data (Rice 1989). We maintained genotype data in a spreadsheet, and then we used the software Convert (Glaubitz 2004) to transform this file into input files for other software packages used in further analyses.

We quantified intrapopulation genotypic variability as mean number of alleles (A), allelic richness (a), and number of private alleles (pa) per locus. We estimated the within-population inbreeding coefficient, F_{IS}, and tested for significant departure from zero with 1,000 randomizations. We performed these analyses with FSTAT 2.9.3 (Goudet 2001). We also estimated effective population size (N_e) for each population using the linkage disequilibrium model method for single sampling efforts implemented in the LDNE program (Waples and Do 2008). This program includes a bias correction from Waples (2006) for uneven sample sizes relative to N_e. We conducted estimates of N_e with parametric confidence intervals (CI) to include alleles with a frequency of ≥ 0.02.

We estimated population differentiation based on microsatellites for comparison to mtDNA estimates using traditional F_{ST} values (Weir and Cockerham 1984); we ascertained significance based on 9,000 randomizations with Monte Carlo simulations and Bonferroni corrections (Rice 1989). We further analyzed genetic structuring with an AMOVA using 9,000 permutations to determine significant deviations from random. We partitioned data in the same manner as the mtDNA AMOVA. We performed IBD tests as described for mtDNA. F_{ST} estimates, AMOVA evaluations, and IBD analyses were carried out using Arlequin ver. 3.1 (Excoffier and others 2005).

We used software Bottleneck (Cornuet and Luikart 1996) to examine evidence for a recent reduction in N_e as suggested by loss of rare allele classes. This program is a coalescent-based method for testing the hypothesis that a recent reduction in effective population size has occurred. We used 9,000 iterations to test the infinite alleles (IAM), stepwise mutation model (SMM), and two-phase model (TPM) with 70-percent SMM and 30-percent variance assuming drift-mutation equilibrium. We tested significance using a one-tailed Wilcoxon signed-rank test ($\alpha = 0.05$) performed in Bottleneck.

RESULTS

Phylogeny

Although *C. mexicanus* is the closest relative to *C. rafinesquii*, a large genetic divergence (> 15 percent; Piaggio and Perkins 2005) between these species was too great to provide any greater statistical reliability for ingroup relationships than midpoint rooting analyses. Therefore, we also generated trees using midpoint rooting. Trees

from both rooting strategies provided the same topology, so we omitted the outgroup to improve readability. We considered 1,064 base pairs from the control region. In the HVII region there was a C-repeat that varied in length among the samples. Often during the sequencing process, the polymerase failed in this repeat region, and determining number of repeats accurately was not possible. Therefore, we eliminated this repeat region across all samples for all analyses. Among the sequences, there were 810 constant sites, 68 variable sites that were parsimony uninformative, and 186 parsimony informative sites. Within the 360 *C. rafinesquii* sequences, there were 318 unique haplotypes. The maximum likelihood tree had 2 statistically supported lineages (clades A and B; figs. 2 and 3) after 1,000 bootstrap iterations. Average uncorrected sequence divergence between these lineages was 4.0 percent (fig. 2). Clade A contained individuals from across the species range, including individuals from each of the five Arkansas colonies (AR1 through AR5) and all other regions sampled (figs. 2 and 3). This clade had no significant bootstrap support (< 50 percent), and there was up to 2-percent sequence divergence within clade A. Clade B's members were only from each of the five known roosts in Arkansas (AR1 through AR5), Texas, and Louisiana. Clade B was well supported with significant bootstrap support
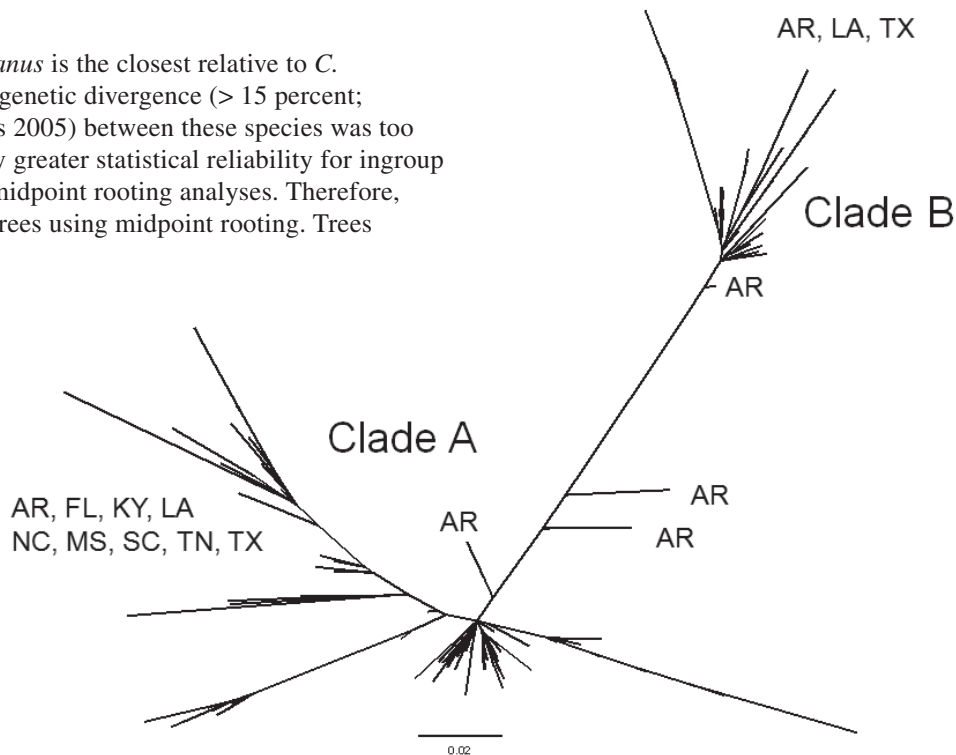

Figure 2—Radial tree layout of maximum likelihood tree inferred from *Corynorhinus rafinesquii* mitochondrial DNA control region. Model parameters of the GTR+G model parameters were estimated and enforced. Both midpoint-rooting and rooting with closest sister taxon strategies provided the same topology, so outgroup taxa were omitted to increase clarity. Samples from across *C. rafinesquii's* range are shown as States where they were collected.
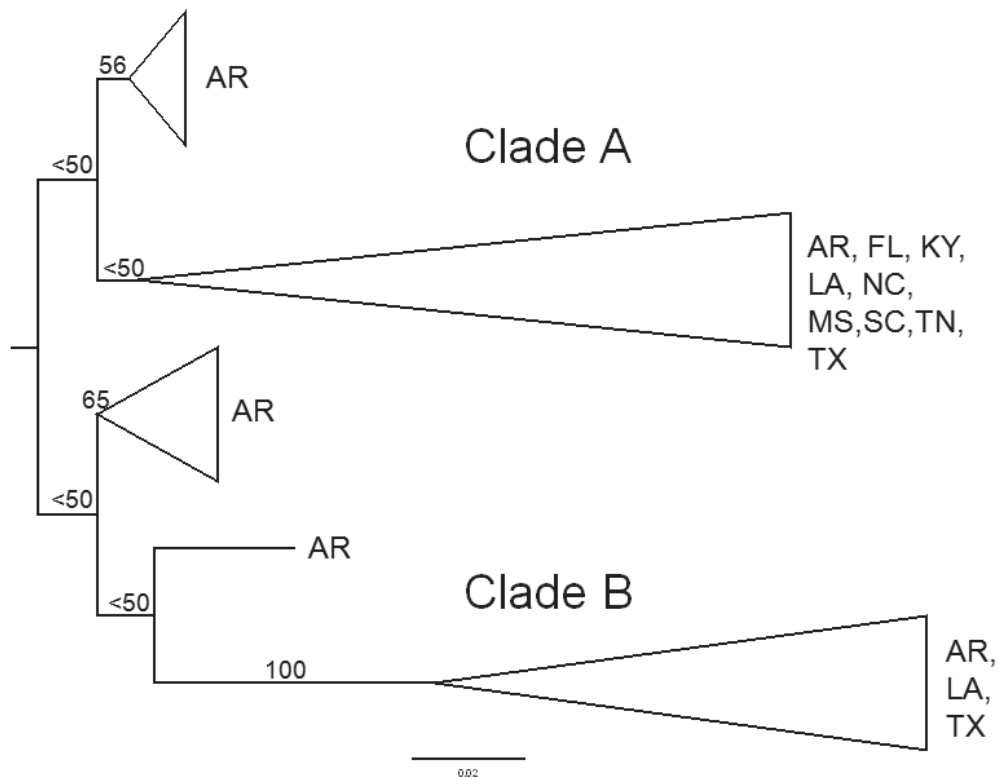
Figure 3—Maximum likelihood phylogram inferred from mitochondrial DNA control region. The GTR+G model parameters were estimated and enforced. Both midpoint-rooting and rooting with closest sister taxon strategies provided the same topology, so in presented trees outgroup taxa were omitted to improve readability. Support for nodes shown as ML bootstrap. Samples of *Corynorhinus rafinesquii* are shown as States where they were collected.

(100 percent), and it had < 1 percent sequence divergence. Lineages that were at least 4 percent divergent comprise the membership within Texas, Louisiana, and each of the five sampled Arkansas populations.

Mitochondrial DNA Sequence Diversity among Populations

The number of unique haplotypes found in each Arkansas colony ranged from 14 to 47, which for each colony is a high proportion of the total haplotypes (table 2). As a result, haplotype diversity was high, ranging from 0.99 to 1.00. Haplotype diversity was also high in other populations (0.87 to 0.99); Louisiana and Texas had the lowest (0.87 to 0.90). Nucleotide diversity was 0.005 to 0.027 within Arkansas colonies, and parsimony informative sites ranged from 45 to 61. The other populations had the same nucleotide diversity (0.005 to 0.024), but number of parsimony informative sites was lower (9 to 46). When examined more closely, Arkansas (except AR3 and AR5), Louisiana, and Texas, which are found in both clades, have at least twice as much nucleotide diversity and parsimony informative sites as North Carolina, Mississippi, and Tennessee populations which are only in clade A. Pairwise differences within only the Arkansas populations were higher (15.73 to 24.62) than the range of pairwise differences within the exclusively clade A populations (4.13 to 8.37). Pairwise differences among colonies in Arkansas were similar to within population differences but ranged lower (18.51 to 26.30) than between Arkansas and any other populations (96.79 to 115.07). Pairwise differences between Texas/Louisiana and North Carolina, Mississippi, and Tennessee populations were similar to within Arkansas (23.43 to 29.27) and lower among North Carolina, Mississippi, and Tennessee populations (6.55 to 19.18). The lowest pairwise differences were between Mississippi and Tennessee populations (6.55 to 7.64).

Pairwise F_{ST} estimates from mtDNA control region sequences ranged from 0.00 to 0.24, and 2 of 10 estimates revealed significant population structure (table 3). The correlation between pairwise genetic differentiation and geographical distance was not significant ($R = 0.15$, $P = 0.11$) among the Arkansas colonies. Across the species range, IBD was significant ($R = 2.5$, $P = 0.006$). The AMOVA suggested that 94 percent of genetic variation was within Arkansas colonies ($P = 0.001$), while the remaining genetic differentiation distributed among populations was significant, albeit low (6 percent; $P = 0.02$).

Table 2—Diversity statistics estimated from mitochondrial DNA control region sequences of five *Corynorhinus rafinesquii* colonies sampled in Arkansas and populations sampled from other locations within the overall range of this species

Pop	N	H	h	SE	π	SE	PI
AR1	48	47	0.999	0.005	0.023	0.001	61
AR2	14	14	1.000	0.027	0.027	0.001	51
AR3	15	14	0.991	0.028	0.005	0.003	45
AR4	29	28	0.998	0.010	0.024	0.012	52
AR5	33	32	0.998	0.008	0.010	0.008	54
LA	17	9	0.904	0.044	0.021	0.011	44
TX	17	7	0.868	0.050	0.024	0.013	46
NC	26	24	0.990	0.015	0.010	0.005	22
MS	16	14	0.983	0.028	0.004	0.003	9
TN (Chester)	31	28	0.994	0.010	0.005	0.003	23
TN (Fentress)	25	21	0.967	0.030	0.005	0.003	14

Pop = population belongs to; N = number of individuals sequenced is reported for each sampling area; H = diversity is measured within colonies or populations as the number of unique haplotypes; h = haplotype diversity; SE = standard error; π = nucleotide diversity; PI = parsimony informative sites; LA = Louisiana; TX = Texas; NC = North Carolina; MS = Mississippi; TN = Tennessee.

Microsatellite Genetic Diversity among Populations

Loci demonstrated linkage equilibria in all pairwise comparisons. There were six significant deviations from HWE after sequential Bonferroni corrections due to lower than expected heterozygosity (table 4). Null alleles can result in departures from HWE. Null alleles were possible in four of five colonies based on Micro-Checker analyses (Van Oosterhout and others 2004). Locus PAUR05 accounted for some of the null allele detections and departures from HWE in three colonies; therefore, we dropped this locus from further analyses. The remaining departures from HWE were found in one colony at locus EF15, in another at Cora_H07F_C05R, and another at locus NN8. We did not drop these loci because they were not out of equilibrium in most of the sampled colonies. Further, three of the departures from HWE and evidence of null alleles were from a single colony, AR3.

Genetic diversity, expressed as number of alleles per locus, ranged from 2 to 16 with the average across loci and colonies being 7.7 (table 5). Average a overall was 4.17, and pa were infrequent, ranging from 0 to 4 per locus and per colony. Average within population expected heterozygosity ranged

Table 3—Pairwise F_{ST} estimated from mitochondrial DNA control region sequences and microsatellite loci for each of the five *Corynorhinus rafinesquii* Arkansas colonies

	AR1	AR2	AR3	AR4	AR5
AR1		0.09	0.01	0.03	0.14[a]
AR2	**0.11**[a]		0.09	0.11	0.24[a]
AR3	**0.001**	**0.15**[a]		0.00	0.07
AR4	**0.04**[a]	**0.26**[a]	**0.00**		0.06
AR5	**0.04**[a]	**0.13**[a]	**0.02**	**0.08**[a]	

Pairwise F_{ST} estimated from mitochondrial DNA are above the diagonal, and estimates from microsatellite DNA are below the diagonal and in boldface type.

[a] $P \leq 0.05$ after Bonferroni corrections.

from 0.56 to 0.59. Inbreeding (F_{IS}) estimated for each colony ranged from 0.06 to 0.18 and was not significantly different from zero except in AR3 and AR4 ($P = 0.05$). Estimated N_e for each colony were low [AR1, 76 (CI 43-218); AR2, –17 (CI 19-∞); AR3, –81 (CI 56-∞); AR4, 19 (CI 11-39); AR5, 24 (CI 13-62)].

Results from the microsatellite DNA AMOVA were similar to the mtDNA AMOVA results; 96 percent of the overall genetic variation found within colonies ($P < 0.001$), while among-population variation was significant (4 percent; $P = 0.002$). The range of pairwise F_{ST} values estimated from microsatellite loci (table 3) was 0.00 to 0.24, comparable to the F_{ST} values estimated from mtDNA. However, a higher number of pairwise comparisons, 7 out of 10, were significantly differentiated. Pairwise linearized F_{ST} estimates from microsatellite DNA were not significantly correlated with log-transformed geographical distances ($R = 0.19$, $P = 0.06$). Therefore, there was no signal of IBD in Arkansas. Finally, there was significant heterozygosity excess detected by the Wilcoxon signed-rank test in two (AR1 and AR4) of the five Arkansas colonies under the IAM but not SMM or TPM in software Bottleneck.

DISCUSSION

Phylogeny

The mtDNA phylogeny (figs. 2 and 3) suggested there are two major divergent lineages within *C. rafinesquii* with an average of 4 percent sequence divergence between them. Our results are consistent with other data from control region, cytochrome *b*, and nuclear DNA sequence data (Lance 1999, Piaggio and Perkins 2005) that indicates a

Table 4—Expected heterozygosity and observed heterozygosity estimated for each microsatellite locus for each *Corynorhinus rafinesquii* Arkansas colony

Pop	AR1	AR2	AR3	AR4	AR5
EF15					
H_o	0.76	0.56	0.31	0.58	0.74
H_e	0.81	0.81	0.82[a]	0.73	0.78
EF21					
H_o	0.70	0.80	0.60	0.45	0.50
H_e	0.55	0.54	0.56	0.38	0.64
EF20					
H_o	0.31	0.63	0.25	0.35	0.37
H_e	0.32	0.73	0.24	0.34	0.32
NN8					
H_o	0.33	0.22	0.07	0.21	0.26
H_e	0.50	0.52	0.52[a]	0.48	0.51
EF14					
H_o	0.02	0.00	0.20	0.10	0.05
H_e	0.02	0.00	0.19	0.10	0.05
Cora_D12_D12					
H_o	0.67	0.44	0.73	0.45	0.28
H_e	0.64	0.58	0.62	0.61	0.34
Cora_E07_E07					
H_o	0.82	0.63	0.73	0.63	0.78
H_e	0.82	0.77	0.77	0.77	0.82
Cora_H07_C05					
H_o	0.36	0.25	0.27	0.37	0.50
H_e	0.58[a]	0.24	0.51	0.63	0.44
Cora_B07_H12					
H_o	0.83	1.00	0.73	0.90	0.89
H_e	0.89	0.88	0.78	0.91	0.88
Cora_E10_G03					
H_o	0.82	0.50	0.93	0.80	0.74
H_e	0.82	0.68	0.85	0.86	0.84

Pop = population belongs to; H_o = observed heterozygosity;

H_e = expected heterozygosity.

[a] Indicates significant departures from Hardy-Weinberg equilibrium (P < 0.05) after sequential Bonferroni corrections (Rice 1989).

lineage (clade B) that is restricted to Arkansas, Louisiana, and Texas, and another lineage (clade A) that is more cosmopolitan and occurs over the entire range of the species. Both clades co-occur in Arkansas, Louisiana, and Texas; and, specifically, both occur within each of the five sampled Arkansas colonies. We did not find these lineages to correlate to subspecies as proposed by Handley (1959). The mtDNA clade comprising only Arkansas, Texas, and Louisiana individuals (clade B) has the best statistical support, suggesting that these bats have been in this region for long enough to allow for this coalescence. Conversely, the mtDNA clade with members from across the range of *C. rafinesquii* (clade A) had no statistical support and shorter branches, suggesting this lineage dispersed more recently into the same region as clade B. Sequence pairwise differences within each Arkansas population were as high as among colonies. Other diversity measures (nucleotide diversity and parsimony informative sites) from Arkansas, Louisiana, and Texas were high when compared to populations that group entirely into clade A. This reflects the two divergent maternal lineages co-occurring within each colony in Arkansas and in regions of Texas and Louisiana. Because both lineages can be found in the same populations, this pattern shows evidence of some maternal structuring but cannot be considered to represent subspecies. Rather, this phylogeographic pattern could indicate that an isolation event or population bottleneck occurred in Arkansas resulting in the loss of clade A in Arkansas and, at a later time, there was another dispersal event or secondary contact (Marjoram and Donnelly 1994) where clade A was reintroduced. Phylogeographic patterns in other taxa suggest existence of a glacial refugium in the Interior Highlands, which includes Arkansas, Louisiana, and Texas, and the eastern highlands (see Mayden 1985, Zamudio and Savage 2003, Zeisset and Beebee 2008). Therefore, it is possible the phylogeographic pattern in *C. rafinesquii* reflects secondary contact between groups that occupied separate refugia, one in the Interior Highlands and the other possibly in the eastern highlands. Alternatively, presence of these divergent clades in the same Arkansas roosts and sampled areas of Texas and Louisiana could suggest multiple dispersal events from one or more source populations. Interestingly, the lowest mtDNA haplotype diversity was found in the Texas and Louisiana populations, but they shared the highest nucleotide diversity measures with Arkansas populations (except AR3 and AR5). Thus, this area harbors haplotypes that are more different from each other than haplotypes from the rest of the range. This may suggest that this area (Arkansas, Louisiana, and Texas) harbors older lineages than in the other sampled regions, and this is supported by the high bootstrap support of clade B (Hewitt 1996, 2000). Conversely, the short branch lengths and low nucleotide diversity coupled with high haplotype diversity within clade A suggest this lineage represents a recent expansion of this lineage which subsequently spread across the current range of *C. rafinesquii*.

Table 5—Diversity estimates and estimated effective population size from microsatellite loci genotyped for individuals from five *Corynorhinus rafinesquii* Arkansas colonies and inbreeding coefficients of each

Pop	F_{IS}	h	N_e	EF15			EF21			EF20			NN8			EF14		
				A	a	pa	A	a	pa	A	a	pa	A	a	pa	A	a	pa
AR1	0.06	0.51+/−0.28	76 (43, 218)	10	5.49	1	3	2.75	0	3	2.49	0	2	2.00	0	2	1.15	0
AR2	0.13	0.33+/−0.20	−17 (19, ∞)	6	5.69	0	3	2.70	0	4	3.88	1	2	2.00	0	1	1.00	0
AR3	0.18*	0.55+/−0.30	−81 (56, ∞)	7	6.10	0	4	3.57	1	3	2.42	0	2	2.00	0	2	1.86	0
AR4	0.17*	0.55+/−0.30	19 (11, 39)	6	4.74	0	3	2.68	0	3	2.32	0	2	2.00	0	2	1.58	0
AR5	0.10	0.53+/−0.29	24 (13, 62)	6	5.23	0	3	2.98	0	3	2.52	0	2	2.00	0	2	1.37	0
All	—	—	—	10	5.85	1	4	3.01	1	4	2.58	1	2		0	3	1.40	0

Pop	Cora_D12_D12			Cora_E07_E07			Cora_H07_C05			Cora_B07_H12			Cora_E10_G03		
	A	a	pa	A	a	pa	A	a	pa	A	a	pa	A	a	pa
AR1	3	2.97	0	9	5.85	1	5	3.19	1	16	8.03	0	11	6.02	2
AR2	3	3.00	0	5	4.75	1	3	2.75	0	7	7.00	0	6	5.50	0
AR3	4	3.43	1	7	5.38	0	5	3.39	0	12	7.30	0	7	6.01	0
AR4	3	2.96	0	6	5.02	0	4	3.22	0	13	8.47	1	10	6.68	1
AR5	3	2.56	0	7	5.47	0	4	2.77	1	13	7.73	0	11	7.11	1
All	4	3.00	1	10	5.69	2	7	3.19	2	18	8.42	1	15	6.60	4

Pop = population belongs to; F_{IS} = the inbreeding coefficient of each colony; diversity estimates are: h = gene diversity averaged across loci; N_e = effective population size; A = number of alleles; a = allelic richness; pa = private alleles; * P = 0.05.

Genetic Diversity within Populations

We predicted that due to habitat loss and subsequent disjunction and/or population reduction, we would detect population bottlenecks in Arkansas *C. rafinesquii* colonies. If true, estimates of genetic diversity and population connectivity would be low, and there might also be inbreeding and low effective population sizes. In fact, we found low genetic diversity across microsatellite loci. We also found significantly high pairwise F_{ST} estimates which indicate low colony connectivity in Arkansas. Further, our results showed that the microsatellite loci were out of short-term linkage equilibrium more than chance would suggest and, without evidence of significant linkage among the loci, revealed low effective population sizes within the last generation for each colony in Arkansas. This, paired with significantly high pairwise F_{ST} estimates from microsatellite data, is surprising over short distances for vagile, volant mammals. High microsatellite pairwise F_{ST} estimates and low N_e along with the detection of two loci very near fixation, with two (NN8) and three (EF14) alleles, can be taken as weak possible evidence of a population bottleneck in Arkansas colonies.

Our analyses detected population bottlenecks in two colonies. However, our data are at the lower limit for number of loci and per population sample size for robust bottleneck detection. Alternatively, it is possible that population bottlenecks have happened more recently than can be detected by these tests. Effective population sizes in AR2 and AR3 were negative, and the CI's included infinity, meaning these are either large populations or the estimate was meaningless. These two populations had the smallest sample sizes and, therefore, may not have allowed robust estimates. Nonsignificant F_{IS} estimates within each population (AR3 and AR4 were both P = 0.05, which may or may not be biologically relevant), and the AMOVAs, which suggested that most variation was attributed to within population differentiation, implies that any population bottleneck has not resulted in inbreeding. Violations of HWE and evidence of null alleles in AR3 may reflect low sample size and/or a Wahlund effect (the sampling of allelic differentiation of two subpopulations within a single sampled colony) due to our samples coming from a hibernaculum where it is possible that multiple unsampled populations may have congregated (Piaggio and others 2009b). However, this does not appear to be the case for AR4 which is also a hibernaculum.

Alternatively, the HWE violations in AR3, lowest mtDNA nucleotide diversity, and parsimony informative sites may be evidence of a recent population bottleneck which was not detected in our bottleneck analysis. Population bottlenecks in Arkansas colonies may also account for the lack of a significant signal of isolation by distance, which suggests there is something other than geography influencing differentiation. Alternatively and more likely, the influence of IBD could have been missed because of low power due to low number of populations sampled. Indeed, significant IBD was detected across the species range where higher sample sizes were obtained. So, either limited sampling in Arkansas accounts for the lack of IBD or there is another factor, i.e., barriers to gene flow, or factors affecting colonies in Arkansas differently than across the species range. In summary, gene flow was restricted among colonies of *C. rafinesquii* in Arkansas, and low effective population sizes suggest that genetic drift is the dominant force on allelic frequencies.

Our within-population diversity estimates from mtDNA and autosomal microsatellites are disparate for each colony in Arkansas. The mtDNA control region sequence diversity was high within colonies and equivalent to the estimated mtDNA control region diversity within populations of the widely distributed migratory bat (*Nyctalus noctula*) (Petit and Mayer 2000). Mitochondrial diversity within *C. rafinesquii* populations was similar or only slightly higher than mtDNA diversity within the sister taxon *C. townsendii* (Piaggio and others 2009b). Conversely, microsatellite genetic diversity within Arkansas colonies was low (Schlötterer and Pemberton 1994) in general. There are several potential explanations for the disparity in our estimates of genetic diversity between mtDNA and microsatellite loci. First, the mtDNA diversity may be large due to occurrence of two divergent lineages within each Arkansas colony. Second, half of the microsatellite markers we used were generated from other bat species (Vespertilionidae: *Eptesicus fuscus*, *Plecotus auritus*, and *N. noctula*) which may pose a problem due to ascertainment bias (Ellergren and others 1995, Webster and others 2002) and result in low estimates of genetic diversity. Third, these two markers are differentially inherited. Autosomal microsatellites are biparentally inherited; whereas, mtDNA is matrilineally inherited and has a smaller effective population size than nuclear DNA. Therefore, demographic processes will affect these markers differently. The different estimates of genetic diversity from mtDNA and microsatellites may then be evidence of very recent and rapid population bottlenecks in Arkansas. Indeed, although haplotype diversity is high in mtDNA, genetic diversity may have been lost (Kuro-o and others 2010). This may be supported by the high nucleotide diversity in some of the Arkansas colonies, which suggests that intermediate haplotypes have been lost. Although there may not be strong evidence of population bottlenecks, microsatellite results

show clear population differentiation among most Arkansas colonies and low effective population sizes.

Implications for Conservation and Management

Based on results of the phylogenetic analysis, it is not appropriate to manage for two subspecies of *C. rafinesquii* as designated by Handley (1959). Rather, it is important to manage and conserve the lineages within *C. rafinesquii* that reflect the evolutionary history of this species. In particular, the lineage with the most limited range (clade B), found only in Arkansas, Louisiana, and Texas, harbors the greatest genetic diversity and includes haplotypes from both lineages.

Over the last 100 to 200 years, bottomland hardwood forests of Arkansas have been systematically cleared of timber, drained, and converted for agricultural use (Dahl 1990, Holder 1970, Woods and others 2004). Today < 10 percent of the original hardwood forests remain. If *C. rafinesquii* relied mostly on these forests for roosts (Clark 1990, 1991), then these bats have experienced habitat destruction and loss of preferred roosts. Further, *C. rafinesquii* in Arkansas now appear to largely occupy human-made structures which are ephemeral and may not provide the long-term habitat necessary to maintain stable populations. We predicted that if habitat loss has resulted in loss of connectivity and/or reduction of populations, then estimates of genetic diversity would be low within the Arkansas colonies. In fact, our estimates of diversity from microsatellite loci is comparable to populations of the federally endangered sister taxon *C. t. virginianus* whose populations are fragmented in four regional populations which are significantly differentiated from each other (Piaggio and others 2009b). This is especially noteworthy given the ongoing problem of loss of manmade structures in Arkansas. For example, AR4 is a hibernaculum in a well that was, until recently, adjacent to an abandoned house used by a maternity roost each summer. A routine check led to the discovery that the house had been demolished. Further, AR1 and AR5 were maternity roosts in abandoned houses, but these houses are now gone. All remaining known roosts of *C. rafinesquii* in Arkansas should be protected and efforts made to identify others and protect those as well.

Dispersal of individuals between populations is critical to maintain population connectivity and genetic diversity, and promoting this is crucial for management or conservation plans. Dispersal produces gene flow over geographic distances. Currently, it appears that dispersal among sampled Arkansas colonies is limited. Further efforts to locate populations of *C. rafinesquii* in remaining bottomland forests and management for forested corridors in bottomlands to provide natural roosts may be needed.

This may help establish connectivity among populations and increase genetic diversity. Without these efforts, colonies of *C. rafinesquii* in Arkansas may be susceptible to disease (Spielman and others 2004), ecological catastrophes, and extinction due to low genetic diversity and small effective population sizes. Finally, comparative studies of populations in other parts of the range are needed to assess whether they also exhibit reduced microsatellite genetic diversity, small effective population sizes, and low connectivity.

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