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Evidence of differential genetic introgression at multiple localities between *Neotoma floridana* and *N. micropus*

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To determine the extent of genetic introgression along the parapatric border between *Neotoma floridana* and *N. micropus*, 140 woodrats were sampled from 21 localities in Kansas, Oklahoma, and Texas, at varying distances from the proposed species boundaries. All individuals were examined at the mitochondrial cytochrome-*b* gene (*Cyt*b) and two nuclear introns: intron seven of the Beta fibrinogen gene (*Fgb*-I7) and intron 2 of the vertebrate alcohol dehydrogenase gene (*Adh*1-I2). Additionally, individuals from a putative contact zone were genotyped using six microsatellite loci to better analyze population structure. Evidence of mixed ancestry was detected in 55 of 140 (39 %) individuals, at 10 of 21 (48 %) localities up to ~150 km from the proposed parapatric boundary. A pattern of differential admixture detected between the two nuclear markers suggested variation in selection pressures at the *Adh*1-I2 and *Fgb*-I7 markers is dependent upon the genomic makeup of the individual. Together, the mitochondrial and nuclear markers indicate evidence of historical hybridization and suggest that hybrid zones within this system are transient in nature.

Para determinar la extensión de la introgresión genética a lo largo del borde parapátrico entre *Neotoma floridana* y *N. micropus*, se tomaron muestras de 140 ratas de campo de 21 localidades en Kansas, Oklahoma y Texas, a diferentes distancias de los límites de las especies propuestas. Todos los individuos fueron examinados en el gen del citocromo-b mitocondrial (Cytb) y dos intrones nucleares: el intrón siete del gen del fibrinógeno Beta (Fgb-I7) y el intrón 2 del gen del alcohol deshidrogenasa de los vertebrados (Adh1-I2). Además, de los individuos de una zona de contacto putativa se obtuvo su genotipo utilizando seis loci de microsatélites para analizar mejor la estructura de la población. Se detectó evidencia de ascendencia mixta en 55 de 140 (39 %) individuos, en 10 de 21 (48 %) localidades hasta ~ 150 km del límite parapátrico propuesto. Un patrón de mezcla diferencial detectado entre los dos marcadores nucleares sugirió una variación en las presiones de selección en los marcadores Adh1-I2 y Fgb-I7 depende de la composición genómica del individuo. Juntos, los marcadores mitocondriales y nucleares indican evidencia de hibridación histórica y sugieren que las zonas híbridas dentro de este sistema son de naturaleza transitoria.

Keywords: Differential introgression; hybridization; microsatellites; parapatry.

Introduction

Two non-sister species of woodrats (*Neotoma floridana* and *N. micropus*; Edwards and Bradley 2002; Longhofer and Bradley 2006) occur parapatrically from the Gulf of Mexico to southeastern Colorado (Figure 1; Hall 1981). Although both species can be found in a variety of habitats, *N. floridana* typically occupies more mesic riparian habitats (Wiley 1980), whereas *N. micropus* generally exploits more shrub-like, xeric habitats (Braun and Mares 1989). The distributions of these two species are separated by a few kilometers at several localities, and by less than one kilometer at others (Spencer 1968; Birney 1973, 1976; Stangl *et al.* 1992; Schmidly 2004; Mauldin *et al.* 2014, 2021). Based on the results of morphologic, allozymic, karyotypic, and genetic data, previous studies determined hybridization occurred along the North Canadian River in Major County, Oklahoma (Spencer 1968; Birney 1973, 1976; Mauldin *et al.* 2014).

Recently, Mauldin *et al.* (2021) examined genotypic variation in individuals collected from Major and Woodward counties and reported that hybridization was intermittent with potentially transient contact zones in this region, as evidence of genetic introgression was present at 11 of 12 sampled localities. Despite this apparent widespread evidence of genetic introgression, only two localities contained mitochondrial DNA (mtDNA) haplotypes of both species and individuals with highly admixed nuclear genomes (Mauldin *et al.* 2021). Additionally, two temporal sampling events (separated by 22 years) from the hybrid zone indicated ongoing and potentially ephemeral hybridization is occurring between the two species in western Oklahoma (Mauldin *et al.* 2021). Similarity of hybrid zone characteristics (*i. e.*, location of the zone, frequency of hybrids detected, directionality of hybridization, level of population substructure detected, etc.) in both datasets

indicated short term stability of the hybrid zone; however, expanded geographic sampling detected varying levels of nuclear admixture at 10 of 11 peripheral localities. Presence of individuals with *N. floridana* mtDNA haplotypes and *N. micropus* nuclear genomes at two localities west of the known area of sympatry suggested the location of the parapatric boundary between these taxa may: 1) be larger than the hybrid zone examined by Mauldin *et al.* (2021) or 2) there may be multiple sites of active hybridization (Mauldin *et al.* 2021).

Evidence of intermittent hybridization in Major County, Oklahoma (Mauldin *et al.* 2014, 2021), has lent support to the possibility that additional areas of hybridization may exist throughout the area of parapatry (Spencer 1968; Birney 1973). A second potential area of contact, along the south bank of the Red River (Locality 20, Figure 1) was sampled at intervals over several years (Stangl *et al.* 1992). Although no morphological evidence of hybridization was reported, Stangl *et al.* (1992) collected *N. floridana* and *N. micropus* within 100 m of each other, thereby establishing the possibility that the two species were in contact. Superficially, this region is similar to that of the known hybrid zone in Major County (Spencer 1968; Birney 1973; Mauldin *et al.* 2014, 2021), as the Red River bisects the parapatric border of these species, and riparian habitat typically exploited by *N. floridana* interdigitates with sage brush and sand dunes, more commonly inhabited by *N. micropus*. In addition to current areas of hybridization, detection of admixed individuals at localities peripheral to the current parapatric boundary could provide insight into the stability of the distributions of these species, and the effect dynamic distributions may have on hybridization in this system.

Given the potential ephemeral nature of the previously studied hybrid zone, along with the long parapatric border shared by these species, Mauldin *et al.* (2021) advocated for further taxonomic sampling along the border of parapatry. They suggested further study was need to determine if 1) additional areas of hybridization exist and 2) evidence of dynamic species distributions could be substantiated. Therefore, the goal of this study was to examine potential areas of sympatry for evidence of hybridization, and to inspect areas peripheral to the parapatric border for evidence of genetic introgression. To this end, multiple objectives were addressed: 1) collect and genotype individuals from localities along and at varying distances from the proposed parapatric border, 2) examine localities for presence or absence of evidence of genetic introgression, 3) determine the maximum recorded distance of hybrid individuals from the current estimated border of parapatry, and 4) utilize microsatellite data to examine population substructure and level of genetic introgression in areas of sympatry.

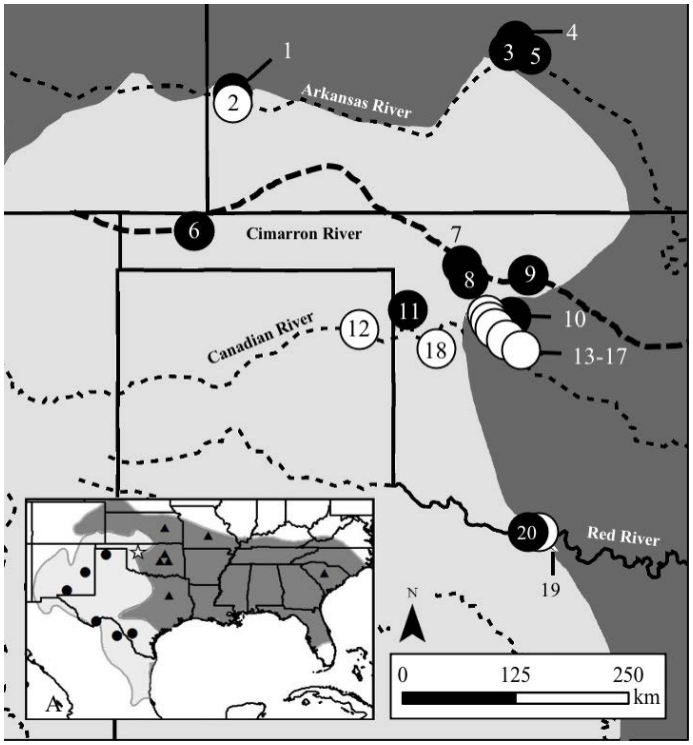

Figure 1. The delineation of the parapatric border that separates the distributions of *N. floridana* and *N. micropus* (shown in dark and light gray, respectively). Collection localities examined in this study are indicated by circles with corresponding locality numbers (Table 1). Localities from which hybrids were detected are shown in black. The white star represents the reported area of hybridization near Seiling, Major County, Oklahoma (Mauldin *et al.* 2014, 2021), of which inset B gives a closeup view. Inset A displays the entire geographic range of both species. For inset A, triangles and circles indicate the collection localities for reference specimens of *N. floridana* and *N. micropus*, respectively.

Materials and methods

Samples. State collecting permits, as well as permission of property owners or appropriate state agencies (*e. g.*, Kansas Department of Wildlife and Parks, Oklahoma Department of Wildlife Conservation, Texas Parks and Wildlife) were received prior to any collection efforts. One hundred and forty woodrats were collected from 21 localities throughout Kansas, Oklahoma, and Texas (Figure 1) between July 2009 and May 2012. Spatial distribution of individual capture sites (middens) were identified with UTM coordinates. Most woodrats were collected with Sherman live-traps (Sherman live-trap Co. Tallahassee, Florida), others were collected with Havahart ® live-traps (Woodstream Corporation, Lititz, Pennsylvania, USA), and some were captured by hand after excavation of middens (nests) to ensure all occupants were collected. Individuals and embryos of sufficient size to ensure extraction of embryonic DNA were given a unique identification number (TK number), sexed, measured, and sacrificed. Individual woodrats were assigned putative species identifications based on morphologic characteristics (Hall 1981; Schmidly 2004), however, given previous results and the inability to distinguish hybrids based solely on morphology, a formal morphological identification based on pelage color was not considered in hybrid identification. Animal care and use guidelines conformed to those proposed by the American Society of Mammalogists (Animal Care and Use Committee 1998) and were approved by the Texas Tech University Institutional Animal Care and Use Committee (IACUC protocol 11009-03). In cases where females and their offspring were captured in the same mid-

den, mother and offspring were cross-referenced; similarly, pregnant females were cross-referenced to embryos. Blood and tissue samples (heart, kidney, liver, lung, muscle, and spleen) were obtained and tissues were immediately frozen in liquid nitrogen, and subsequently archived at the Natural Science Research Laboratory (NSRL) at the Museum of Texas Tech University. Voucher specimens (skulls, postcranial skeletons, and skins) were prepared and deposited in the NSRL (Appendix 1). Additionally, liver samples of four woodrats (indicated by prefix TJM in Tables A1 and A2) were obtained from the lab of Ivan Castro-Arellano at Texas State University.

DNA Isolation. Total genomic DNA (nuclear and mitochondrial) was isolated from each individual using approximately 0.1 g of liver and the Qiagen DNeasy kit (Qiagen Inc.; Valencia, California, USA). In some cases, entire embryos were required to isolate sufficient DNA. DNA samples were stored at -20˚C for subsequent analyses.

Genotype Analyses. All genotype analyses followed the protocol outlined in detail by Mauldin *et al.* (2014, 2021). Eight *N. floridana* and seven *N. micropus* collected a minimum distance of 125 km from the parapatric border, and previously utilized by Mauldin *et al.* (2014, 2021) were included as reference samples (Figure 1; Appendix 1). Three loci were examined, two autosomal loci (intron two of the vertebrate alcohol dehydrogenase gene (*Adh*1-12) and intron seven of the beta-fibrinogen gene (*Fgb*-I7)) and one mitochondrial DNA locus (Cytochrome-*b, Cyt*b). Additionally, individuals from Locality 20 were genotyped for six microsatellite loci (*Nma*01, *Nma*04, *Nma*05, *Nma*06, *Nma*10, and *Nma*11) developed by Castleberry *et al.* (2000) to detect genetic structure within the population.

Adh1-12 Assay. A banding pattern unique to *N. floridana* was produced using the restriction enzyme *Nsi*I (ATGCA/T) with a fragment of the *Adh*1-12 region of either 566 bp or 390 bp that had been amplified using PCR methods modified from Amman *et al.* (2006) and Longhofer and Bradley (2006) using one of the following primer pairs: ExonII-F and 2340-II (566 bp product) or 350F and 2340-II (390 bp product; Amman *et al.* 2006). Restriction digests were conducted following manufacturer's methods and are outlined in Mauldin *et al.* (2014).

Fgb-I7 *Assay*. Mauldin *et al.* (2014) reported that although no restriction enzyme was diagnostic, three diagnostic nucleotide substitutions were identified (positions 428, 497, and 493). Therefore, sequence data was collected on a 609-610 bp fragment amplified using PCR primers *Fgb*-I7L-*Rattus* and *Fgb*-I7U-*Rattus* from Wickliffe *et al.* (2003) and following PCR methods modified from Prychitko and Moore (2000) as outlined in Carroll and Bradley (2005). Sequence data has been deposited in GenBank (Appendix 1). Though previous studies have utilized this as a diagnostic marker (Mauldin *et al.* 2014, 2021), it is possible unsorted polymorphisms not detected in reference samples may exist.

*Cyt*b *Assay*. The entire *Cyt*b gene was amplified using two PCR primers (LGL765 forward—Bickham *et al.* 1995 and LGL 766 reverse—Bickham *et al.* 2004) and conditions outlined in Edwards and Bradley (2002). The restriction enzyme [*Bsa*I (GGTCTC(N)$_1$/)] produced a cut that was unique to *N. floridana* following methods outlined by the manufacturer and reported by Mauldin *et al.* (2014, 2021).

Microsatellite Assay. The six microsatellite loci developed by Castleberry *et al.* (2000) and utilized by Mauldin *et al.* (2014, 2021) were amplified and analyzed for all individuals collected at Locality 20 (Figure 1) as described by Haynie *et al.* (2007). Alleles were scored using GeneMapper software (version 4.0; Applied Biosystems Inc.).

Data Analysis. Based on the results of molecular assays outlined above, each individual was scored as either *N. micropus* or *N. floridana* for the mitochondrial genome, and as homozygous *N. micropus*, heterozygous, or homozygous *N. floridana* for the *Adh*1-I2 and *Fgb*-I7 markers. GenAlEx (version 6.5; Peakall and Smouse 2012) was utilized to identify presence of duplicate genotypes, test microsatellite loci for deviation from Hardy-Weinberg equilibrium (HWE) expectations, and format data for use in Structure (v2.3.4; Pritchard *et al.* 2000) as codominant nuclear markers with only adults and subadults being included in the analyses. Mitochondrial data were not analyzed in Structure or NewHybrids (*v.1.1Beta3*; Anderson and Thompson 2002) but were included in result plots to aid in identification of hybrid individuals and examine any potential bias present in directionality of introgression.

Based on preliminary results (nuclear introgression and geographic proximity of both mtDNA haplotypes), complete nuclear genotypes (*Adh*1-I2, *Fgb*-I7, and six microsatellite loci) for all individuals collected at Locality 20 were analyzed in Structure to examine population structure and quantify potential admixture between the two species. Structure runs utilized the admixture model with independent allele frequency option, a burnin of 500,000, run length of 1,000,000 iterations, and examined values of K (clusters) from 1-5. Two separate parameter sets were run, one assigned reference individuals to a priori populations using the popflag designation (parameter set A), whereas the other did not (parameter set B). Neither dataset used prior population assignment information for study samples. Structure result files were uploaded to Structure Harvester (Earl and vonHoldt 2012) to determine the value of K which best fit the data using the Evanno method (Evanno *et al.* 2005).

Results of the Structure run with the smallest variance value from parameter set A (K = 2) were used to generate a plot for examination of admixture between the two species. Furthermore, individuals from Locality 20 were analyzed in NewHybrids to determine the posterior probability values (PPVs) of individuals belonging to one of six classifications (pure parental *N. floridana*, pure parental *N. micropus*, F_1, F_2, backcross to *N. floridana*, backcross to *N. micropus*) based on admixture of nuclear genomes with no prior allele frequency data, Uniform priors, a burnin of 100,000, and 1,000,000 sweeps after burnin. Structure and NewHybrids output files were visualized using Excel

2010 (Microsoft Corporation, Redmond, Washington, USA). Assignment to hybrid classifications followed the protocol outlined by Mauldin *et al.* (2014, 2021).

Electronic species distributions of *N. floridana* and *N. micropus* (Patterson *et al.* 2007) generated by digitizing previously published range maps (*i. e.*, Hall 1981) were used to approximate the location of the parapatric border. Distance of each sampling locality to the closest point along the approximated parapatric boundary was then measured with the use of ArcGIS Software (ESRI, Redlands, California, USA), based on UTM coordinates of localities. Distances were measured to each distributional boundary (*N. floridana* and *N. micropus*) along the same vector, and the two distances were averaged for the final estimate. Additionally, samples from Locality 20 were collected from two nonadjacent parcels of private property; however, given the proximity of localities (all samples collected within ~2.5 km), samples from both properties were consolidated into a single locality for simplicity. However, these localities are examined both jointly (Locality 20) and independently (Localities 20a and 20b) to better examine patterns of interspecific genetic introgression at multiple scales.

Randomization tests of goodness-of-fit utilized 20,000 iterations and were conducted with Excel 2010 (Microsoft Corporation, Redmond, Washington, USA) following methods described by McDonald (2009) to determine if the following proportions deviated significantly from an equal contribution: 1) proportion mtDNA haplotypes of each species at Localities 20, 20a, and 20b, 2) proportion of *Adh*1-I2 and *Fgb*-I7 alleles detected at localities within each presumed species distribution, and 3) proportion of *Adh*1-I2 and *Fgb*-I7 alleles detected east and west of the proposed center of the hybrid zone in Major County, Oklahoma (data from Mauldin *et al.* 2014). The proportion test within the statistical package R (Team 2008) was utilized to compare the following proportions: *N. floridana* mtDNA haplotypes detected at Localities 20a and 20b, hybrid individuals with introgression detected at the *Adh*1-I2 locus within the distributions of *N. micropus* and *N. floridana*, respectively, and hybrid individuals with introgression detected at the *Fgb*-I7 locus within the distributions of *N. micropus* and *N. floridana*, respectively.

Results

Results of molecular assays are available in Appendix 2. Evidence of mixed ancestry was detected in 55 of 140 (39 %) sampled individuals, at 10 of 21 (48 %) localities (Figure 1). A high percentage of individuals with mixed ancestry (>50 %) was recorded at three localities (4, 9, and 20). Genetic introgression was detected at both nuclear loci at only two localities (9 and 20), whereas only Locality 20 contained mitochondrial DNA (mtDNA) haplotypes of both species. Given that Locality 20 is the only site at which both mtDNA haplotypes were detected, and the possibility that it represents an area of current or recent contact (Stangl *et al.* 1992), individuals from Locality 20 were not included in examination of differential detection of admixture between loci. Of the 22 woodrats with mixed ancestry collected within the distributional limits of *N. micropus*, evidence of nuclear admixture was detected at the *Adh*1-I2 locus in one animal, and at the *Fgb*-I7 locus in 21 individuals ($P < 0.0001$). Admixture was detected only at the *Adh*1-I2 locus for all 21 admixed individuals identified within the distribution of *N. floridana* ($P < 0.0001$). No individuals were heterozygous at both loci. A similar bias was identified through use of randomization test of goodness-of-fit that examined data from the Major County hybrid zone, as detection of admixture in individuals collected west of the proposed center of the zone was significantly biased towards *Fgb*-I7 locus ($P = 0.012$), and detection of admixture east of the zone was biased, although not significantly, to the *Adh*1-I2 locus ($P = 0.073$). The furthest distance from the parapatric border at which nuclear admixture was detected was approximately 150 km within the species distribution of *N. micropus* (Locality 6; Figure 1). Additional distance data for localities and individuals is available in Table 1.

Examination of microsatellite data with GenAlEx identified no duplicate genotypes. The following markers was determined to deviate significantly from HWE expectations within the sampled population, *Nma*05 in Locality 20b ($P = 0.030$). Results of Structure and Structure Harvester analyses determined K = 2 as the most appropriate number of clusters for both parameter sets. Results of Structure analyses detected no genetic introgression or population substructure at Locality 20 (Figure 2). Results of NewHybrids analyses of samples from Locality 20 identified only one sample as less than 90 % probability of belonging to the classification of 'pure' *N. micropus* (Figure 3; TK179266 = 87.76 %; mean *N. micropus* PPV = 96.56 %, median *N. micropus* PPV = 98.77 %). Spatial distribution of mtDNA haplotypes within Locality 20 is depicted in Figure 4. Results of randomization test of goodness-of-fit determined the proportion of mtDNA haplotypes of each species present at Locality 20 did not vary significantly from the null model of equal contribution ($P = 0.118$), nor did Locality 20a ($P = 0.690$); however, Locality 20b was significantly biased towards *N. floridana* mtDNA, with no *N. micropus* mtDNA haplotypes detected ($P = 0.004$). Results of analyses of the proportion of *N. floridana* mtDNA haplotypes at Locality

Table 1. The mean, median, minimum, and maximum distances (in kilometers) of each category from the closest point of the estimated parapatric border.

Category	Mean	Median	Minimum	Maximum
All localities	42	27	4	152
hybrid localities	45	29	12	152
'pure' localities	40	27	4	152
all individuals	34	26	4	152
hybrid individuals	27	26	12	152
'pure' individuals	38	26	4	152

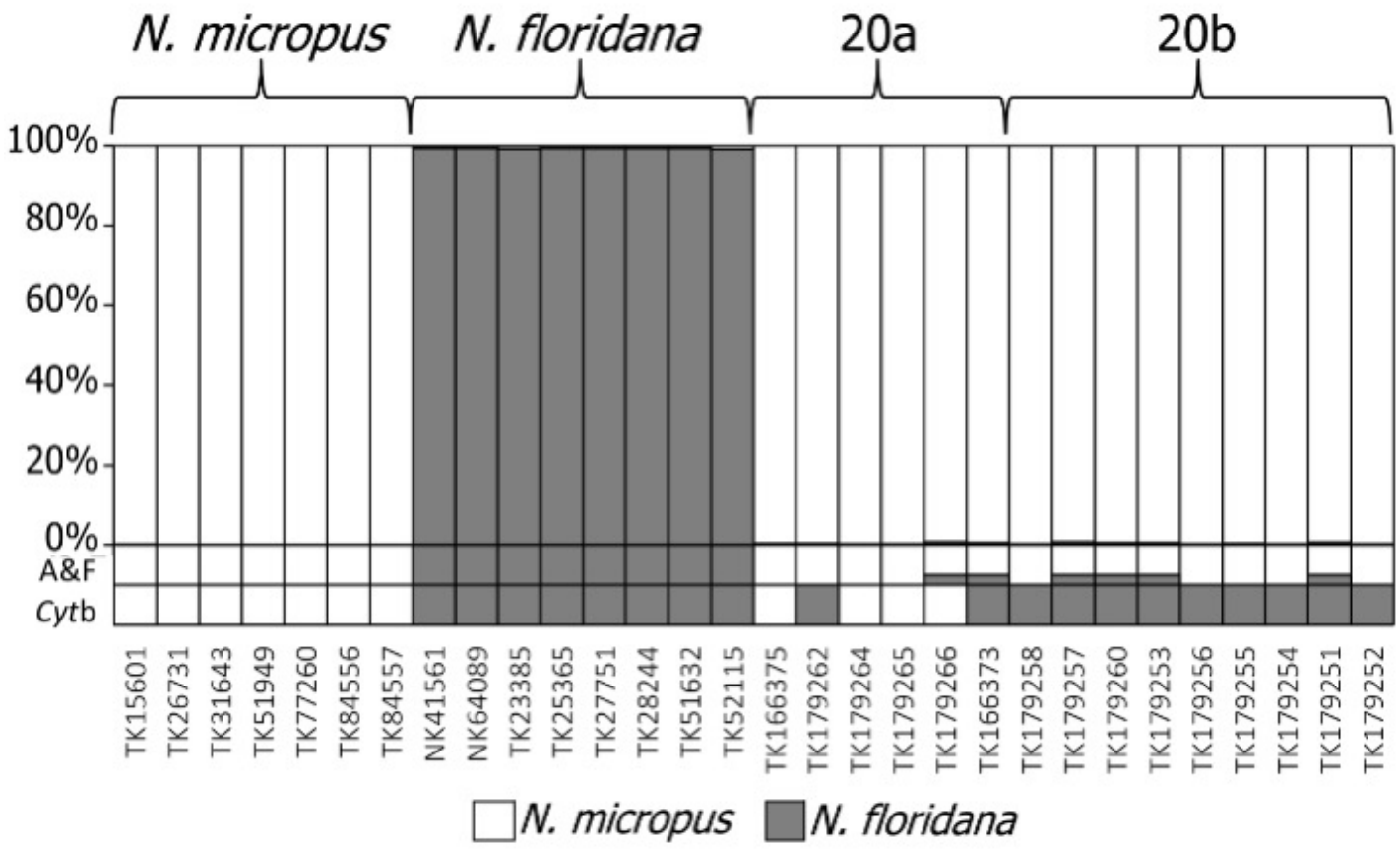

Figure 2. Results of Structure analyses: genotype information for all individuals collected from Locality 20. Specimen identification numbers are shown below the respective bar. Shading signifies the proportion of alleles contributed by each species (White: *N. micropus*, Gray: *N. floridana*). The top tier denotes the estimated proportion of the specimen's microsatellites as determined by Structure analyses, the second tier (*A&F*) indicates the proportion of *Adh*1-I2 and *Fgb*-I7 alleles, the third tier (*Cyt*b) identifies the mtDNA haplotype of the individual. Brackets and labels indicate reference samples of each species, and collection localities for all study individuals.

20b were significantly higher than that of Locality 20a ($P = 0.024$). Results of analyses of the proportion of hybrids with introgression detected at the *Adh*1-I2 ($P < 0.0001$) and *Fgb*-I7 ($P < 0.0001$) loci varied significantly depending upon the distribution from which they were collected.

Discussion

A high proportion of sampled woodrats (39 %) were determined to be of mixed ancestry, including individuals from 10 of 21 (48 %) sampled localities throughout Texas, Oklahoma, and Kansas (Appendix 2). Given the small number of molecular markers examined, these values likely underestimate the true number of genetically admixed individuals and localities at which they are found. These results suggest some degree of hybridization has occurred, or currently occurs, at multiple localities along the parapatric border. Additionally, at three localities (4, 9, and 20) greater than 50 % of examined individuals exhibited some level of genetic introgression; although no genetic admixture was detected at three localities (13, 14, and 19) of similar or lesser distances to the border. Furthermore, the geographic distance between some sampled hybrids and the putative location of the parapatric boundary is substantial (*e. g.*, Locality 6: ~150 km).

Finally, the locus at which admixture was detected was dependent upon the species distribution from which the samples were collected. For individuals collected within the distribution of *N. micropus*, genetic introgression was detected most frequently at the *Fgb*-I7 locus; however, for individuals collected within the distribution of *N. floridana*, introgression was detected only at the *Adh*1-I2 marker. The statistically significant difference in the locus at which exotic alleles were detected within each species distribution suggests that selection favors inclusion of foreign DNA sequences at different loci based upon the genomic background of the organism (*i. e.*, predominantly *N. floridana* or *N. micropus* nuclear genomes). Examination of the Major County hybrid zone data generated by Mauldin *et al.* (2014) identified a similar bias at a smaller geographic scale.

Results of Structure analyses failed to detect nuclear admixture at Locality 20 and estimated that nuclear genomes of sampled woodrats were predominantly (>99 %) composed of *N. micropus* alleles, although the majority contained *N. floridana* mtDNA haplotypes. Examination of results of NewHybrids analyses in combination with *Adh*1-I2, *Fgb*-I7, and *Cyt*b data determined all individuals from Locality 20 were either backcrosses to *N. micropus* (12) or putatively pure *N. micropus* (3), with no *N. floridana* parental types

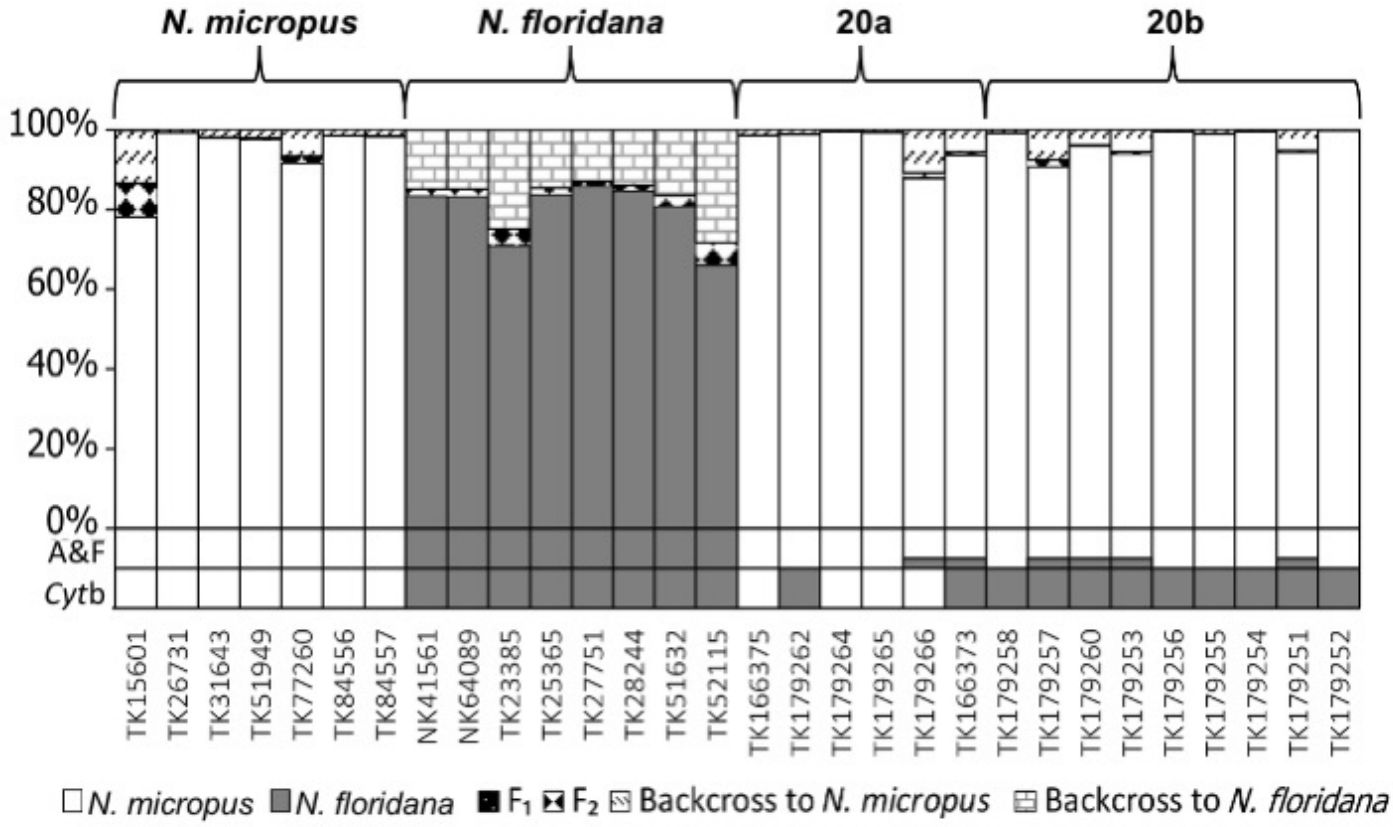

Figure 3. Results of NewHybrids analyses: genotype information for all individuals collected from Locality 20. The posterior probability that the individual belongs to a specified hybrid classification (*i. e.*, pure parental, F_1, F_2, etc.) based on microsatellite data is indicated by the proportion of each color or pattern within the top tier. The second tier (*A&F*) indicates the proportion of *Adh*1-I2 and *Fgb*-I7 alleles, and the third tier (*Cytb*) identifies the mtDNA haplotype of the individual. Brackets and labels indicate reference samples of each species or collection localities of specimens for this study.

detected. The statistically significant change in proportion of mtDNA haplotypes present between Localities 20a and 20b, combined with paucity of *N. floridana* parental types, and nuclear genomes of all individuals composed primarily of *N. micropus* alleles suggests the location of the hybrid zone has shifted from the approximate location of Locality 20a to some location east of the sampled area. The easternmost sample (TK 179251) was collected ~100 m east of the I-44 Bridge reported to be an area of contact (Stangl *et al.* 1992) and appeared to be *N. micropus* (pelage and nuclear genome). Therefore, it is possible that the shift in area of sympatry began prior to the study by Stangl *et al.* (1992), at which time it had reached the I-44 Bridge, and has subsequently continued east along the Red River, with the mtDNA haplotype of *N. floridana* occurring throughout its now displaced range.

Similar cytonuclear discordance, although smaller and directionally reversed, was reported between the positions of the mitochondrial and nuclear boundaries between these species in Major County, Oklahoma, suggesting that the areas of hybridization are somewhat transient as distributional borders of these species shift over generations (Mauldin *et al.* 2021). Given the large variation in degree of genetic introgression detected over relatively small distances and the small proportion of individuals detected with highly admixed nuclear genomes (F_1 and F_2-like individuals) reported along the North Canadian River (Mauldin *et al.* 2021), distance to the putative area of sympatry cannot be estimated with any certainty. However, it is worth noting that an individual identified as a putatively pure *N. floridana* was collected from Locality 19 (~10 km east of Locality 20) in Oklahoma.

Various methodologies, including morphologic, karyotypic, allozymic, and genotypic data have been used to examine hybridization at various geographic scales within this system (Spencer 1968; Birney 1973, 1976; Mauldin *et al.* 2014, 2021). Examination of these studies and the research presented herein has determined the following characteristics are demonstrative of hybridization between these species: a high percentage of genetically admixed individuals, no evidence of reduced fertility in hybrid individuals, a paucity of F_1 and F_2-like genotypes, significant linkage disequilibrium, limited population structure, differential genetic introgression of nuclear loci, and varying levels of hybrid zone ephemerality. Examination of these characteristics in the framework of mechanistic models of hybrid zone maintenance and criteria set forth by Endler (1977) and Moore (1977), as summarized by Van Den Bussche *et al.* (1993), indicate that either the hybrid equilibrium model (wherein hybrids and parental types are equally fit) or the

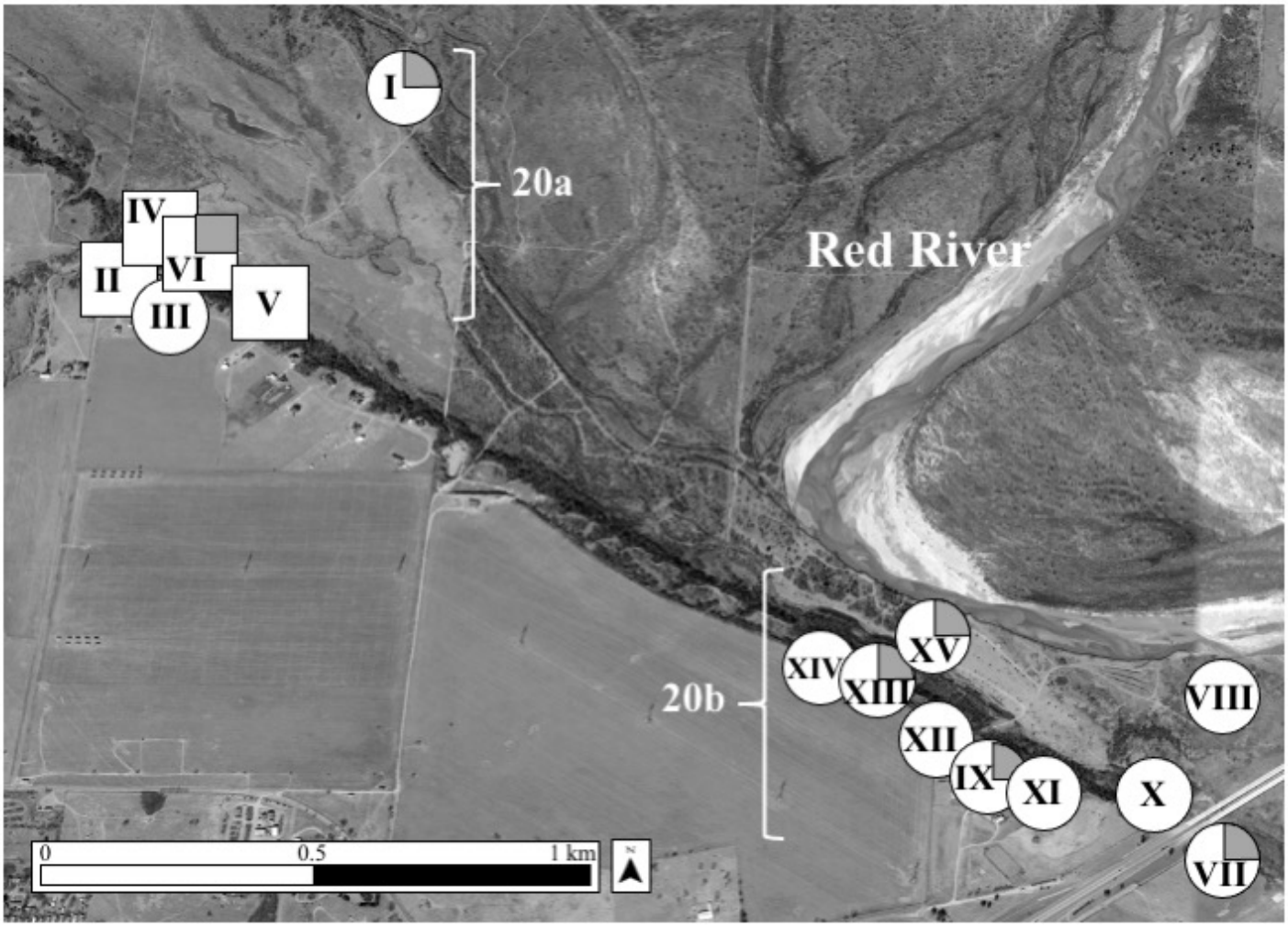

Figure 4. Map depicting Locality 20 near the Red River in Wichita County, Texas. Numbers signify midden locality and shape (Circles: *Neotoma floridana* and Squares: *N. micropus*) indicates the mtDNA haplotype (*Cyt*b) detected at each midden site. Shading represents the proportion of *Adh*1-I2 and *Fgb*-I7 alleles contributed by each species (Gray: *Neotoma floridana* and White: *Neotoma micropus*). No evidence of introgression was identified based on the microsatellite data (see Figure 2) at this locality; however, genotypes based on the combination of the *Cyt*b, *Adh*, and *Fgb* markers indicated that introgression occurred at all middens except 2, 4, and 5. For reference, the highway/bridge in the lower right corner is Interstate-44.

hybrid superiority model (in which hybrids have higher fitness within an ecotone or certain set of environmental conditions) might be responsible for maintaining hybridization between these species. Additional data concerning hybrid fitness, selection pressures, and possible correlations to environmental conditions are needed to distinguish between these models relative to the dynamics responsible for maintaining hybrid zones between these species.

Historical distribution changes of *N. floridana* have been documented by Quaternary fossil records (Richards 2013), 'recent fossil' remains dating to the late Holocene (~1,450 years before present – Eshelman 1971; Richards 2013), and temporal sampling in the 19th century (Cope 1872; Blatchley 1897). Additionally, a study examining distributions of *N. micropus* and *N. albigula* in southern New Mexico, determined interspecific competition led to displacement of *N. micropus* over a portion of the study area (Wright 1973). Given the evidence presented herein, the ephemeral nature of distributional boundaries, and the documented occurrence of interspecific displacement within the genus *Neotoma*, it is possible that the evidence of introgression detected at peripheral localities is a result of some combination of distributional shifts and dispersal of alleles over generations. Subsequently, the differential detection of alleles at distinct nuclear loci might be the result of disparity in persistence of certain alleles within populations, the rate at which those same alleles disperse over generations, or some combination thereof. Additionally, the possibility of unsorted polymorphisms may exist, potentially impacting the nuclear introgression calculations; however as all but one of the molecularly identified hybrids exhibited cytonuclear discordance, this would not change the overall results or the classification for most animals examined.

In conclusion, nuclear introgression was detected at multiple localities throughout a large portion of the parapatric border including sites near Burkburnett, Texas, Seiling, Oklahoma, as well as Great Bend and Syracuse, Kansas, among others (see black dots in Figure 1). Additionally, this introgression appears to be variable with regard to prevalence of admixture detected at separate nuclear markers

dependent upon the genomic background of the organism, as the *N. micropus* genome appears to tolerate *N. floridana* alleles at the *Fgb*-I7 locus better than at the *Adh*1-I2 locus, and *N. floridana* genome is more commonly infiltrated with *N. micropus* alleles at the *Adh*1-I2 locus than the *Fgb*-I7 locus. The presence of cytonuclear discordance at Locality 20, and similar evidence reported in Major and Woodward counties (Mauldin *et al.* 2021) provide evidence of nuclear genome displacement, likely caused by distributional shifts. Although introgression appears common throughout the parapatric border, the differential introgression of alleles and paucity of individuals determined to have highly admixed nuclear genomes, suggest hybridization does not pose a major threat to the gene pools of either species.

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Dedication. This manuscript is dedicated to Dr. David J. Schmidly for his devotion to mammalogy and the training of graduate students. I (RDB) first met Dr. Schmidly in the fall of 1982, while enrolled in his mammalogy course. His mammalogy class was one of the most challenging courses I had as an undergraduate, and I loved it! Dr. Schmidly asked me to work on a M.S. degree, under his tutelage, and it was the best professional decision I could have made. In the summer of 1983, I accompanied Dr. Schmidly and his NSF research team to Mexico, where we spent 6 weeks collecting specimens of *Peromyscus* for his research project. That summer changed my life and I was hooked on mammalian systematics. Later, Dr. Schmidly insisted that I go to Texas Tech University and obtain a PhD with the late Dr. Robert J. Baker; I would not have been confident enough to do so without Dave's encouragement. Joining me on the author-line are two of my former PhD students (MLH and MRM), as well as a current PhD student (SCV) who received her MS from MLH, so the tradition of training graduate students in mammalogy continues! Dave, given that this paper pertains to woodrats and hybridization, two topics on which you have several publications, we hope we have well-represented your teachings. On a personal note, I thank you for all you have done for me and my students over the years. You set the bar high and we continue to try and keep up with you!!!

Literature cited

Amman, B. R., J. D. Hanson, L. K. Longhofer, S. R. Hoofer, and R. D. Bradley. 2006. Intron 2 of the alcohol dehydrogenase gene (*Adh*1-I2): a nuclear DNA marker for mammalian systematics. Occasional Papers, Museum of Texas Tech University 256:1–16.

Anderson, E. C., and E. A. Thompson. 2002. A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160:1217–1229.

Animal Care and Use Committee. 1998. Guidelines for the capture, handling, and care of mammals as approved by the American Society of Mammalogists. Journal of Mammalogy 79:1416–1431.

Bickham, J. W., J. C. Patton, D. A. Schlitter, I. L. Rautenbach, and R. L. Honeycutt. 2004. Molecular phylogenetics, karyotypic diversity, and partition of the genus *Myotis* (Chiroptera: Vespertilionidae). Molecular Phylogenetics and Evolution 33:333–338.

Bickham, J. W., Wood, C. C., and J. C. Patton. 1995. Biogeographic implications of cytochrome *b* sequences and allozymes in sockeye (*Oncorhynchus nerka*). Journal of Heredity 86:140–144.

Birney, E. C. 1973. Systematics of three species of woodrats (genus *Neotoma*) in central North America. Miscellaneous Publications of the Museum of Natural History – University of Kansas Publications 58:1–173.

Birney, E. C. 1976. An assessment of relationships and effects of interbreeding among woodrats of the *Neotoma floridana* species group. Journal of Mammalogy 57:103–132.

Blatchley, W. S. 1897. Indiana caves and their fauna. 21st Annual Reports of the Department of Geology and Natural Resources of Indiana for 1896. p. 177.

Braun, J. K., and M. A. Mares. 1989. *Neotoma micropus*. Mammalian Species 330:1–9.

Carroll, D. S., and R. D. Bradley. 2005. Systematics of the genus *Sigmodon*: DNA sequences from Beta-Fibrinogen and Cytochrome *b*. Southwestern Naturalists 50:342–349.

Castleberry, S. B., T. L. King, P. B. Wood, and W. M. Ford. 2000. Microsatellite DNA markers for the study of Allegheny woodrat (*Neotoma magister*) populations and cross-species amplification in the genus *Neotoma*. Molecular Ecology 9:817–829.

Cope, E. D. 1872. On the Wyandotte Cave and its fauna. Nature 6:406–422.

Earl, D. A., and B. M. von Holdt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359–361.

Edwards, C. W., and R. D. Bradley. 2002. Molecular systematics of the genus *Neotoma.* Molecular Phylogenetics and Evolution 25:489–500.

Endler, J. A. 1977. Geographic variation, speciation, and clines. Princeton (NJ): Princeton University Press, U.S.A.

Eshelman, R. E. 1971. The paleoecology of Willard Cave, Delaware County, Iowa. MS Thesis, University Iowa, Iowa City, U.S.A.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology. 14:2611–2620.

Hall, E. R. 1981. The mammals of North America. 2nd vol. :John Wiley and Sons. New York, U.S.A.

Haynie, M. L., S. G. Bennett, M. Rood, B. Hess, C. F. Fulhorst, and R. D. Bradley. 2007. Genetic variation in multilocus microsatellite genotypes in two species of woodrats (*Neotoma macrotis* and *N. fuscipes*) from California. Journal of Mammalogy 88:745–758.

Longhofer, L. K., and R. D. Bradley. 2006. Molecular systematics of the genus *Neotoma* based on DNA sequences from intron 2 of the alcohol dehydrogenase gene. Journal of Mammalogy 87:961–970.

Mauldin, M. R., M. L. Haynie, J. D. Hanson, R. J. Baker, and R. D. Bradley. 2014. Multi-locus characterization of a woodrat (genus *Neotoma*) hybrid zone. Journal of Heredity 105:466-476.

Mauldin, M. R., M. L. Haynie, S. C. Vrla, and R. D. Bradley. 2021. Temporal evaluation of a woodrat (genus *Neotoma*) hybrid zone based on genotypic and georeferenced data. Journal of Mammalogy DOI:10.1093/jmammal/gyaa164

McDonald, J. H. 2009. Handbook of Biological Statistics (2nd ed.). Baltimore (MD) USA: Sparky House Publishing.

Moore, W. S. 1977. An evaluation of narrow hybrid zones in vertebrates. The Quarterly Review of Biology 52:263-277.

Patterson, B. D., G. Ceballos, W. Sechrest, M. F. Tognelli, T. Brooks, L. Luna, P. Ortega, I. Salazar, and B. E. Young. 2007. Digital distribution maps of the mammals of the western hemisphere, version 3.0. NatureServe, Arlington. Virginia, U.S.A.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28:2537–2539.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Prychitko, T. M., and W. S. Moore. 2000. Comparative evolution of the mitochondrial Cytochrome b gene and nuclear b-Fibrinogen intron 7 in Woodpeckers. Molecular Biology and Evolution 17:1101–1111.

Richards, R. L. 2013. Quaternary distribution of the eastern woodrat, *Neotoma floridana*, in Indiana. Proceedings of the Indiana Academy of Science 96:513–521.

Schmidly, D. J. 2004. The Mammals of Texas. University of Texas Press. Austin, U.S.A.

Spencer, D. L. 1968. Sympatry and hybridization of the eastern and southern plains wood rats. Unpub. PhD thesis. Stillwater (OK): Oklahoma State University.

Stangl, F. B. Jr., W. W. Dalquest, and R. J. Baker. 1992. Mammals of southwestern Oklahoma. Occasional Papers of the Museum of Texas Tech University 151:1–47.

Team, RDC. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from http://www.r-project.org

van Den Bussche, R. A., R. K. Chesser, M. J. Hamilton, R. D. Bradley, C. A.Porter, and R. J. Baker. 1993. Maintenance of a narrow hybrid zone in *Peromyscus leucopus*: a test of alternative models. Journal of Mammalogy 74:832–845.

Wickliffe, J. K., F. G. Hoffman, D. S. Carroll, Y. V. Dunina-Barkovskaya, R. D. Bradley, and R. J. Baker. 2003. PCR and sequencing primers for intron 7 (*Fgb*-I7) of the fibrinogen, B beta polypeptide (*Fgb*) in mammals: a novel nuclear DNA marker. Occasional Papers of the Museum of Texas Tech University 219:1–6.

Wiley R. W. 1980. *Neotoma floridana*. Mammalian Species 139:1–7.

Wright M. E. 1973. Analysis of habitats of two woodrats in southern New Mexico. Journal of Mammalogy 54:529–535.

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Appendix 1

Specimens examined– A comprehensive list of all specimens examined for this study. Specimen identification numbers (TK – Museum of Texas Tech University; NK – Museum of Southwestern Biology, University of New Mexico; TJM = collection number of specimens from Texas State University) followed by *Cyt*b, *Adh*1-I2, and *Fgb*-I7 GenBank accession numbers (*e. g.*, Museum ID # – *Cyt*b GenBank #, *Adh*1-I2 GenBank #, *Fgb*-I7 GenBank #). All specimens are from the United States unless noted otherwise.

Reference samples:

*Neotoma floridana***–** United States; Kansas; Lyon County, Ross Natural History Preserve, 4 mi W, 1 mi S Americus (TK28244 – AF186818, AY817640, DQ180021); Missouri; Pulaski County, Fort Leonard Wood (NK41561 – AF294333, KF860899, KF861009); Oklahoma; Creek County, Heyburn State Recreation Area (TK27751 – AF294341, AY817639, DQ180020); Oklahoma; McIntosh County, 3.1 mi E Dustin (TK23385 – AF294339, EU284810, KF861010); Pottawatomie County, 2.5 mi N, 5.9 mi E Tecumseh (TK25365 – AF294340, KF860901, KF861011); South Carolina; Richland County, Congaree Swamp NM (NK64089– AF294335, AY817637, DQ180054); Texas; Anderson County, Gus Engeling Wildlife Management Area (TK52115 – AF294344, KF860902, KF861012); Brazoria County, Peach Point Wildlife Management Area (TK51632– AF294343, KF860903, KF861013).

*Neotoma micropus***–** Mexico; Coahuila, 20 mi S Morelos (TK16501 – AF186824, KF860904, KF861014); United States; New Mexico; Otero County, Ft. Bliss Military Base (TK77270 – AF376474, AY817653, DQ180049); Roosevelt County, 16.5 mi S, 3 mi E Taiban (TK31643 – AF186822, AY817652, DQ180048); Texas; Brewster County, Black gap Wildlife Management Area (TK51949 – AF298845, KF860905, KF861015); Dimmit County, Chapparral Wildlife Management Area (TK84556 – AF186826, AY817654, DQ180050; TK84557– AF186827, AY817655, DQ180040); Moore County, 4 mi N, 1 mi E Dumas (TK26731 – EU286808, EU284813, KF861016).

Specimens from study sites: (Museum ID number, *Fgb*-I7 GenBank Accession number)

Locality 1.--- Kansas; Hamilton County, 1.5 mi N, 2.0 mi W Syracuse, Hamilton Co. Wildlife Management Area, (TK175812 – KJ611149; TK175813 – KJ611150; TK175814 – KJ611151; TK175815 – KJ611152; TK175816 – KJ611153; TK175818 – KJ611154; TK175819 – KJ611155)

Locality 2.— Kansas; Hamilton County, 0.5 mi S, 3.6 mi W Syracuse (Girlscout Camp: TK175806 – KJ611146; TK175807 – KJ611147; TK175808 – KJ611148)

Locality 3.— Kansas; Barton County; Cheyenne Bottoms Wildlife Area (TK165470 – KJ611062; TK165471 – KJ611063; TK165472 – KJ611064; TK165473 – KJ611065; TK165474 – KJ611066; TK165475 – KJ611067; TK165476 – KJ611068; TK165477 – KJ611069; TK165479 – KJ611070; TK165480 – KJ611071; TK165481 – KJ611072; TK165483 – KJ611073; TK169501 – KJ611128; TK169503 – KJ611129; TK169504 – KJ611130; TK169505 – KJ611131; TK169506 – KJ611132; TK169690 – KJ611137; TK169691 – KJ611138; TK169694 – KJ611139)

Locality 4.— Kansas; Barton County; 3.5 mi N Great Bend (TK169598 – KJ611133; TK169599 – KJ611134; TK169600 – KJ611135; TK169601 – KJ611136)

Locality 5.— Kansas; Barton County; 1.0 mi S, 0.2 mi W Ellinwood (TK175771 – KJ611140; TK175772 – KJ611141; TK175773 – KJ611142; TK175774 – KJ611143; TK175775 – KJ611144; TK175776 – KJ611145)

Locality 6.— Oklahoma, Cimarron County; Black Mesa State Park (TK160982 – KJ611043; TK163031 – KJ611044)

Locality 7.— Oklahoma; Woodward County, Boiling Springs State Park (TK167362 – KJ611104; TK167363 – KJ611105; TK167369 – KJ611106; TK167434 – KJ611121)

Locality 8.— Oklahoma; Woodward County, 2 mi S, 6 mi E Woodward (TK167500 – KJ611124; TK168001 – KJ611125; TK168007 – KJ611126; TK168009 – KJ611127)

Locality 9.— Oklahoma; Major County, 5 mi W Cleo Springs (TK167392 – KJ611107; TK167393 – KJ611108; TK167395 – KJ611109; TK167396 – KJ611110; TK167405 – KJ611111; TK167406 – KJ611112; TK167413 – KJ611113; TK167414 – KJ611114; TK167415 – KJ611115; TK167416 – KJ611116; TK167417 – KJ611117; TK167418 – KJ611118; TK167419 – KJ611119; TK167420 – KJ611120; TK167451 – KJ611122; TK167452 – KJ611123)

Locality 10.— Oklahoma; Dewey County, 1 mi N, 9 mi E Seiling (Canton WMA: TK167337 – KJ611089; TK167339 – KJ611090; TK167346 – KJ611091; TK167347 – KJ611092; TK167348 – KJ611093; TK167349 – KJ611094; TK167350 – KJ611095; TK167351 – KJ611096; TK167353 – KJ611097; TK167354 – KJ611098; TK167355 – KJ611099; TK167356 – KJ611100; TK167357 – KJ611101; TK167360 – KJ611102; TK167361 – KJ61110): Oklahoma; Blaine County, 2.9 mi S Canton Lake Recreational Area - Big Bend Campground (TK160840 – KJ611033; TK160841 – KJ611034; TK160843 – KJ611035; TK160845 – KJ611036; TK160846 – KJ611037; TK160847 – KJ611038; TK160849 – KJ611039; TK160850 – KJ611040; TK160851 – KJ611041; TK160865 – KJ611042)

Locality 11.— Oklahoma; Ellis County, Ellis Co. Wildlife Management Area (TK165342 – KJ611047; TK165382 – KJ611049; TK165383 – KJ611050; TK165384 – KJ611051; TK165385 – KJ611052; TK165386 – KJ611053; TK165387 – KJ611054; TK165388 – KJ611055; TK165389 – KJ611056; TK165390 – KJ611057)

Locality 12.— (Texas, Hemphill County, Gene Howe Wildlife Management Area, (TK165429 – KJ611058; TK165430 – KJ611059; TK165437 – KJ611060; TK165455 – KJ611061)

Locality 13.— Oklahoma; Dewey County, 6 mi N, 4 mi W Oakwood (TK166466 – KJ611083; TK166467 – KJ611084; TK166491 – KJ611086)

Locality 14.— Oklahoma; Dewey County, 1 mi S, 2.5 mi E Taloga (TK166493 – KJ611087; TK166494 – KJ611088)

Locality 15.— Oklahoma; Dewey County, 3 mi N, 6 mi W Oakwood (TK166441 – KJ611081)

Locality 16.— Oklahoma; Dewey County, 2 mi N, 7 mi W Oakwood (TK166402 – KJ611077; TK166403 – KJ611078; TK166404 – KJ611079; TK166405 – KJ611080)

Locality 17.— Oklahoma; Dewey County, 0.2 mi N, 0.5 mi W Fay (TK166462 – KJ611082; TK166474 – KJ611085)

Locality 18.— Oklahoma; Roger Mills County, 10.0 mi N, 2.5 mi W Cheyenne, Black Kettle National Grassland (TK165310 – KJ611045; TK165335 – KJ611046; TK165365 – KJ611048)

Locality 19.— Oklahoma; Cotton County, 5.5 mi S, 1mi E Randlett (TK166379 – KJ611076)

Locality 20a.— Texas; Wichita County, 1 mi N Burkburnett (TK166373 – KJ611074; TK166375 – KJ611075; TK179262 – KJ611165; TK179264 – KJ611166; TK179265 – KJ611167; TK179266 – KJ611168)

Locality 20b.— Texas; Wichita County, 0.5 mi N, 1 mi E Burkburnett(TK179251 – KJ611156; TK179252 – KJ611157; TK179253 – KJ611158; TK179254 – KJ611159; TK179255 – KJ611160; TK179256 – KJ611161; TK179257 – KJ611162; TK179258 – KJ611163; TK179260 – KJ611164;)

Locality 21.— Texas; Bastrop County, 10 mi S, 5 mi W Rosanky (TJM151 – KJ611169; TJM650 – KJ611170; TJM658 – KJ611171; TJM679 – KJ611172)

Appendix 2

Identification, demographic, locality, and genetic assay data for each individual woodrat examined in this study. Abbreviations are as follows: ID# = Unique identification number (TK = NSRL field identification number, TJM = collection number of specimens from Texas State University); sex: m = male, f = female, u = unknown sex; age: A = Adult, SA = Subadult, J = Juvenile, E = Embryo; genotype: M = homozygous for *N. micropus* alleles at the respective locus, F = homozygous for *N. floridana* alleles at the respective locus, H = heterozygous at the respective locus; Class = final classification of the individual: hyb = hybrid individual, mic = putatively pure *N. micropus* individual, flor = putatively pure *N. floridana* individual. Superscripts after TK numbers indicate the family unit (a-f) to which the individual belongs.

ID#	Sex	Age	Locality #	Adh1-I2	Cytb	Fgb-I7	Class
TK175812	f	A	1	M	M	H	hyb
TK175813	f	A	1	M	M	M	mic
TK175814	f	A	1	M	M	F	hyb
TK175815	m	A	1	M	M	M	mic
TK175816	m	A	1	M	M	H	hyb
TK175818	m	A	1	M	M	M	mic
TK175819	m	A	1	M	M	M	mic
TK175806	f	A	2	M	M	M	mic
TK175807	f	SA	2	M	M	M	mic
TK175808	f	A	2	M	M	M	mic
TK165470	f	A	3	H	F	F	hyb
TK165471	m	A	3	F	F	F	flor
TK165472	f	A	3	F	F	F	flor
TK165473	m	A	3	F	F	F	flor
TK165474	f	SA	3	F	F	F	flor
TK165475	f	A	3	F	F	F	flor
TK165476	f	A	3	F	F	F	flor
TK165477	f	A	3	H	F	F	hyb
TK165479	m	A	3	M	F	F	hyb
TK165480	f	SA	3	H	F	F	hyb
TK165481	m	A	3	H	F	F	hyb
TK165483	f	A	3	H	F	F	hyb
TK169501	f	A	3	F	F	F	flor
TK169503	f	A	3	F	F	F	flor
TK169504	f	A	3	F	F	F	flor
TK169505	f	A	3	F	F	F	flor
TK169506	f	A	3	F	F	F	flor
TK169690	f	SA	3	H	F	F	hyb
TK169691	f	A	3	H	F	F	hyb
TK169694	m	A	3	H	F	F	hyb
TK169598	f	A	4	H	F	F	hyb
TK169599	f	A	4	H	F	F	hyb
TK169600	m	A	4	H	F	F	hyb
TK169601	m	A	4	H	F	F	hyb
TK175771	f	A	5	F	F	F	flor
TK175772	f	A	5	F	F	F	flor
TK175773	m	A	5	F	F	F	flor
TK175774	f	A	5	F	F	F	flor
TK175775	m	A	5	F	F	F	flor
TK175776	m	A	5	F	F	F	flor
TK160982	f	SA	6	M	M	H	hyb
TK163031	f	SA	6	M	M	M	mic
TK167362	f	SA	7	M	M	M	mic
TK167363	m	A	7	M	M	M	mic
TK167369	f	A	7	M	M	H	hyb
TK167434	f	A	7	M	M	M	mic
TK167500	f	SA	8	M	M	M	mic
TK168001	f	A	8	M	M	M	mic
TK168007	f	A	8	M	M	M	mic
TK168009	f	A	8	M	M	H	hyb
TK167392	m	A	9	M	M	M	mic
TK167393	m	J	9	M	M	H	hyb
TK167395	m	J	9	M	M	H	hyb
TK167396[a]	f	A	9	M	M	F	hyb
TK167405[b]	f	A	9	M	M	H	hyb
TK167406	m	A	9	M	M	M	mic
TK167413	f	A	9	M	M	F	hyb
TK167414[a]	u	E	9	M	M	H	hyb
TK167415[a]	u	E	9	M	M	H	hyb
TK167416[a]	u	E	9	M	M	H	hyb
TK167417[a]	u	E	9	M	M	H	hyb
TK167418[a]	u	E	9	M	M	H	hyb
TK167419[b]	u	E	9	M	M	H	hyb
TK167420[b]	u	E	9	M	M	H	hyb
TK167451[c]	f	A	9	H	M	M	hyb
TK167452[c]	m	J	9	M	M	H	hyb
TK160840	m	A	10	F	F	F	flor
TK160841	f	A	10	H	F	F	hyb
TK160843	m	A	10	H	F	F	hyb
TK160845	m	A	10	M	F	F	hyb
TK160846	f	A	10	F	F	F	flor
TK160847	f	A	10	F	F	F	flor
TK160849	f	SA	10	F	F	F	flor
TK160850	f	A	10	H	F	F	hyb
TK160851	f	J	10	F	F	F	flor
TK160865	f	A	10	F	F	F	flor
TK167337	f	SA	10	F	F	F	flor
TK167339	m	SA	10	F	F	F	flor
TK167346	f	A	10	F	F	F	flor
TK167347	f	SA	10	F	F	F	flor
TK167348	f	A	10	F	F	F	flor
TK167349	f	A	10	M	F	F	hyb
TK167350	m	A	10	F	F	F	flor
TK167351	f	A	10	F	F	F	flor

Appendix 2

Continuation...

ID#	Sex	Age	Locality #	Adh1-I2	Cytb	Fgb-I7	Class
TK167353[d]	f	A	10	F	F	F	flor
TK167354	f	A	10	F	F	F	flor
TK167355	u	SA	10	M	F	F	hyb
TK167356	m	A	10	M	F	F	hyb
TK167357	f	SA	10	M	F	F	hyb
TK167360[d]	u	E	10	F	F	F	flor
TK167361[d]	u	E	10	F	F	F	flor
TK165342	m	SA	11	M	M	H	hyb
TK165382	f	A	11	M	M	M	mic
TK165383	f	A	11	M	M	M	mic
TK165384	f	A	11	M	M	M	mic
TK165385	m	A	11	M	M	M	mic
TK165386	f	A	11	M	M	M	mic
TK165387	f	A	11	M	M	H	hyb
TK165388	f	A	11	M	M	M	mic
TK165389	f	A	11	M	M	M	mic
TK165390	f	A	11	M	M	M	mic
TK165429	f	A	12	M	M	M	mic
TK165430	f	A	12	M	M	M	mic
TK165437	f	A	12	M	M	M	mic
TK165455	m	A	12	M	M	M	mic
TK166466	m	A	13	F	F	F	flor
TK166467	m	A	13	F	F	F	flor
TK166491	f	A	13	F	F	F	flor
TK166493	f	A	14	F	F	F	flor
TK166494	f	A	14	F	F	F	flor
TK166441	f	A	15	F	F	F	flor
TK166402	f	A	16	F	F	F	flor
TK166403	m	A	16	F	F	F	flor
TK166404	f	A	16	F	F	F	flor
TK166405	f	A	16	F	F	F	flor
TK166462[e]	f	A	17	F	F	F	flor
TK166474[e]	u	E	17	F	F	F	flor
TK165310	m	A	18	M	M	M	mic
TK165335	f	SA	18	M	M	M	mic
TK165365	f	A	18	M	M	M	mic
TK166379	m	A	19	F	F	F	flor
TK166373	m	A	$20a_{I}$	H	F	M	hyb
TK166375	m	A	$20a_{II}$	M	M	M	mic
TK179262	m	A	$20a_{III}$	M	F	M	hyb
TK179264	m	SA	$20a_{IV}$	M	M	M	mic
TK179265[f]	f	A	$20a_{V}$	M	M	M	mic
TK179266[f]	u	E	$20a_{VI}$	M	M	H	hyb
TK179251	f	A	$20b_{VII}$	M	F	H	hyb
TK179252	f	J	$20b_{VIII}$	M	F	M	hyb
TK179253	m	J	$20b_{IX}$	H	F	M	hyb
TK179254	f	A	$20b_{X}$	M	F	M	hyb
TK179255	f	SA	$20b_{XI}$	M	F	M	hyb
TK179256	f	A	$20b_{XII}$	M	F	M	hyb
TK179257	f	A	$20b_{XIII}$	H	F	M	hyb
TK179258	f	A	$20b_{XIV}$	M	F	M	hyb
TK179260	f	A	$20b_{XV}$	H	F	M	hyb
TJM151	f	A	21	F	F	F	flor
TJM650	m	A	21	F	F	F	flor
TJM658	m	A	21	F	F	F	flor
TJM679	f	SA	21	F	F	F	flor