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Comprehensive genetic analyses reveal evolutionary distinction of a mouse (*Zapus hudsonius preblei*) proposed for delisting from the US Endangered Species Act

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Abstract

***Zapus hudsonius preblei*, listed as threatened under the US Endangered Species Act (ESA), is one of 12 recognized subspecies of meadow jumping mice found in North America. Recent morphometric and phylogenetic comparisons among *Z. h. preblei* and neighbouring conspecifics questioned the taxonomic status of selected subspecies, resulting in a proposal to delist the *Z. h. preblei* from the ESA. We present additional analyses of the phylogeographic structure within *Z. hudsonius* that calls into question previously published data (and conclusions) and confirms the original taxonomic designations. A survey of 21 microsatellite DNA loci and 1380 base pairs from two mitochondrial DNA (mtDNA) regions (control region and cytochrome *b*) revealed that each *Z. hudsonius* subspecies is genetically distinct. These data do not support the null hypothesis of a homogeneous gene pool among the five subspecies found within the southwestern portion of the species' range. The magnitude of the observed differentiation was considerable and supported by significant findings for nearly every statistical comparison made, regardless of the genome or the taxa under consideration. Structuring of nuclear multilocus genotypes and subspecies-specific mtDNA haplotypes corresponded directly with the disjunct distributions of the subspecies investigated. Given the level of correspondence between the observed genetic population structure and previously proposed taxonomic classification of subspecies (based on the geographic separation and surveys of morphological variation), we conclude that the nominal subspecies surveyed in this study do not warrant synonymy, as has been proposed for *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius*.**

Keywords: control region, cytochrome *b*, microsatellite, phylogeography, subspecies, *Zapus hudsonius*

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Introduction

Zapus hudsonius preblei is one of 12 recognized subspecies of meadow jumping mice found throughout North America (Hafner *et al*. 1981). The distribution of *Z. h. preblei* is confined to the riparian systems where moisture is most plentiful (Jones *et al*. 1983). At present, the subspecies' range stretches along the eastern slope of the Rocky Mountains from east-central Wyoming south to Colorado Springs, Colorado. The availability of suitable riparian habitat is declining throughout the range of *Z. h. preblei* due to degradation caused by agricultural, residential, and commercial development (US Fish & Wildlife Service 1998). As a result of diminishing suitable riparian habitat and small population sizes, *Z. h. preblei* was listed as a threatened species under the US Endangered Species Act (ESA) in 1998 (US Fish and Wildlife Service 1998).

Effective programs for conserving threatened taxa require the identification of unambiguous units of management that reflect evolutionarily important lineages. The issue of defining appropriate units of management becomes acute

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when protection of a taxon under consideration relies on proof of distinction. Subspecies recognition within *Z. hudsonius* has been based primarily on geographic disjunction and morphological variation (Krutzsch 1954; Hafner *et al*. 1981) among relatively recently radiated populations. Given that morphological comparisons among accepted species of the genus *Zapus* have failed to provide reliably diagnostic characters (Jones 1981; but see Conner & Shenk 2003), it stands to reason that these malleable characters may not prove unfailing at distinguishing intraspecific taxa. Thus, the morphologically similar subspecies of *Z. hudsonius* present significant challenges to conservation biologists because of taxonomic uncertainty (Preble 1899; Krutzsch 1954; Ramey *et al*. 2005). Taxonomy based solely on morphological characters may not be consistent with phylogenetic relationships, as the rate of evolutionary change can vary among lineages and similar environmental influences may cause convergence (Grant 1987). This can complicate resource management efforts and ultimately jeopardize the ecological and evolutionary potential of a lineage (Moritz & Hillis 1996).

An integrative conservation approach that identifies and sustains ecological processes and evolutionary lineages is needed to protect and manage the biodiversity present in the southwestern portion of *Z. hudsonius*' range. Inherent in such an approach is the identification and characterization of associated migration, colonization, and extinction processes among populations of these putative subspecies (Avise 2004). Molecular markers, with a clear heritable genetic basis and the number of characters limited only by genome size (Moritz & Hillis 1996), provide insight into these processes and can be used to reveal genetic discontinuities and distinctiveness among or between taxa with subtle or undetectable morphological differentiation (Clark *et al*. 2000). Patterns of gene exchange, the extent of genealogical relationships, and accurate reflections of true evolutionary relationships (i.e. phylogeny) can be revealed through the use of the appropriate type and number of molecular genetic markers (Moritz & Hillis 1996; Avise 2004).

Recent morphological and genetic comparisons among *Z. h. preblei* and neighbouring con-specifics have questioned the taxonomic status of several subspecies (Ramey *et al*. 2005, REA). The multidisciplinary study utilized a hypothesis testing approach to determine uniqueness of subspecies, including analyses of cranial morphometrics and contemporary genetic techniques. REA reported that multivariate morphometric analyses on selected cranial measurements failed to support the original description of *Z. h. preblei* as a distinct subspecies (but see Vignieri *et al*. 2006). Genetic components of the study compared haplotypes within the mitochondrial DNA (mtDNA) control region and multilocus genotypes at nuclear microsatellite DNA loci. REA concluded that recent gene exchange and low levels of genetic structure among subspecies supported synonymization of *Z. h. preblei*, *Z. h. campestris* (Bear Lodge meadow jumping mouse), and *Z. h. intermedius* (meadow jumping mouse). REA's critical test of uniqueness for *Z. h. preblei* and related taxa was that greater variance be demonstrated between subspecies than within, a test criterion that, to our knowledge, has not been fully evaluated for taxonomical, biological, or statistical relevance with molecular data. REA constitutes the lone published molecular population genetic analysis of *Z. hudsonius*, with important implications for the evolution, ecology, and conservation status of *Z. h. preblei*. The proposed synonymy of these subspecies has prompted a subsequent reevaluation of the status of *Z. h. preblei* under the ESA (US Fish and Wildlife Service 2005).

Studies of phylogeographic relationships among intraspecific taxa often exact a more rigorous study design than that required for interspecific comparisons (Avise 2004). Further, phylogeographic studies can be initially misled by dependence on tissues yielding insufficient quality (Kirchman *et al*. 2001) and consistency of DNA (Steinberg 1999), inadequate portrayals of genealogical relationships (Brower *et al*. 1996) through use of inappropriate methodology (Posada & Crandall 2001), or insufficient resolution from too few molecular or morphological characters (Smouse & Chevillon 1998). REA utilized dried museum skins from selected collections, assessed the differentiation among selected subspecies of *Z. hudsonius* with a hierarchical pairwise haplotypic distance approach, surveyed sequence variation for a 346 base pair (bp) fragment of the mtDNA control region, and represented nuclear DNA variation with five microsatellite DNA loci. In light of the unproven distinctiveness criteria applied to a relatively small fragment of mtDNA and minimal number of microsatellite loci, the methods applied and conclusions drawn by REA warrant independent verification.

We report additional results concerning the phylogeographic structure and evolutionary distinctiveness of *Zapus hudsonius* subspecies, placing particular emphasis on the relatedness of *Z. h. preblei* to each neighbouring subspecies by comparing a larger collection of samples over a greater representation of both the mitochondrial and nuclear genomes than was previously investigated. Analyses consisted of evaluating sequence variation at approximately 374 bp of the mtDNA control region and 1006 bp of the mtDNA cytochrome *b* region, combined with the fragment analysis of a fourfold greater number of nuclear microsatellite DNA loci than surveyed by REA. We tested the null hypothesis that collections of *Z. h. preblei*, *Z. h. campestris*, *Z. h. intermedius*, *Z. h. pallidus*, and *Z. h. luteus* comprise a single homogeneous unit (i.e. these subspecies fail to exhibit genetic discreteness) as reflected in the spatial distribution of mtDNA haplotypes and microsatellite DNA allele frequencies. Due to the taxonomic revision proposed by REA, importance was placed on comparisons among

Table 1 Sample size, allelic richness, number of private alleles, observed and expected heterozygosity, and estimates of F_{IS} observed for 14 collections of *Zapus hudsonius* representing five neighbouring subspecies surveyed at 21 microsatellite DNA loci

Subspecies	Collection abbreviation	Collection locality	Cluster*	N	*A*†	Private alleles	H_O	H_E	F_{IS}
Z. h. preblei				170	6.7	[5]	0.539	0.624	0.136
	SOWY	Laramie, Albany, Platte, and Converse Counties, WY		(28)	4.7	(1)	0.499	0.553	0.099
	LCCO1	N. Fk. Cache la Poudre River, Larimer County, CO		(14)	3.4	(2)	0.507	0.502	–0.010
	LCCO2	Stove Prairie Creek, Larimer County, CO	*Z. h. preblei*	(16)	3.4		0.536	0.528	–0.015
			— North	58	5.4		0.511	0.614	0.169
	DCCO1	East Plum Creek, Douglas County, CO		(34)	4.3		0.538	0.535	–0.005
	DCCO2	Indian Creek, Douglas County, CO		(30)	4.0	(1)	0.538	0.540	0.004
	ECCO1	US Air Force Academy, El Paso County, CO		(22)	4.4	(1)	0.563	0.584	0.037
	ECCO2	Monument Creek, El Paso County, CO	*Z. h. preblei*	(26)	4.0		0.586	0.559	–0.050
			— South	112	5.7		0.553	0.583	0.052
Z. h. campestris			*Z. h. campestris*	61	7.2	[2]	0.637	0.670	0.051
	CCWY	Beaver Creek, Crook County, WY		(30)	6.2		0.648	0.662	0.021
	CCSD	Iron/Willow Creeks, Custer/ Pennington Counties, SD		(31)	6.0	(1)	0.625	0.654	0.045
Z. h. intermedius			*Z. h. intermedius*	49	9.4	[22]	0.649	0.703	0.079
	BRCSD	Columbia Road Reservoir, Brown County, SD		(28)	6.1	(8)	0.619	0.637	0.029
	MCMN	Camp Ripley, Morrison County, MN		(21)	8.3	(13)	0.687	0.735	0.067
Z. h. pallidus			*Z. h. pallidus*	48	9.4	[33]	0.752	0.790	0.049
	BCSD	Cedar Creek, Bennett County, SD		(16)	6.9	(9)	0.738	0.757	0.026
	KBCNE	N. Channel Platte River, Kearney/Buffalo Counties, NE		(32)	7.5	(12)	0.759	0.789	0.038
Z. h. luteus	SCNM	Multiple sites, Sandoval County, NM	*Z. h. luteus*	20	4.6	[8]	0.576	0.623	0.076

WY, Wyoming; CO, Colorado; SD, South Dakota; MN, Minnesota; NE, Nebraska; NM, New Mexico.
*Clusters ($k = 6$) were determined using the program STRUCTURE.
†Allelic richness.
‡[ ] indicates the number of private alleles limited to 1 or all collections within a subspecies.

Z. h. preblei, *Z. h. campestris*, and *Z. h. intermedius*. In the present study, we accepted as evidence of subspecific distinctiveness the conditions previously defined as significant phylogeographic separation of mtDNA alleles between subspecies (or populations), combined with congruent phylogeographic structure for nuclear loci (Avise & Ball 1990; Ball & Avise 1992; Moritz 1994a).

Methods

Minimally invasive tissue samples (e.g. ear punches and blood) of *Zapus hudsonius campestris*, *Z. h. intermedius*, *Z. h. pallidus*, and *Z. princeps* were obtained from individuals trapped in summer 2005 (Table 1). Archived tissue samples were obtained from Z. *h. preblei* (ear punch), *Z. h. intermedius* (frozen liver) and *Z. h. luteus* (frozen liver) (Appendix A). In order to validate the haplotype data reported by REA as being shared among disjunct and/or highly differentiated subspecies, tissue from 15 specimens was obtained from the University of Kansas Natural History Museum, Lawrence, KS (KUNHM) 11 *Z. h. campestris*, two *Z. h. interemedius*, and two *Z. h. pallidus*; Appendix B. These specimens represent seven of the 10 haplotypes reported as being shared among subspecies by REA (haplotypes C/P1, C/P2, C/P3, C/P4, C9/INT-VII, L/PAL/C1, and L/PAL/C2). All tissues were forwarded directly from the collector or museum to the US Geological Survey-Leetown Science Center, Kearneysville, West Virginia for analysis. DNA was obtained from ear punches (in 95% ethanol), frozen (–80 °C) liver, or blood tissue (on FTA cards; Whatman Inc., Clifton, NJ, USA) and extracted using the PUREGENE DNA extraction kit (Gentra Systems) and resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was obtained from dried museum skin sections using the DNeasy Tissue Extraction Kit (QIAGEN) with the manufacturer's protocol modified as described in Iudica *et al*. (2001).

Microsatellite DNA amplification and fragment analysis

Twenty-one microsatellite loci developed by three different laboratories were screened in all individuals sampled (Appendix C). The three sets of markers were: (i) *Z*.7, *Z*.20, *Z*.26, *Z*.48, and *Z*.52 (REA); (ii) *Ztri*2, *Ztri*17, *Ztri*19, and *Ztri*24 (isolated from *Z. trinotatus*; Vignieri 2003); and (iii) *Zhu*C3, *Zhu*C6, *Zhu*C12, *Zhu*C104, *Zhu*C119, *Zhu*C120, *Zhu*C129, *Zhu*C130, *Zhu*D107, *Zhu*D108, *Zhu*D109, and *Zhu*D122 (King *et al*. 2006). Multiple requests by the US Geological Survey, Leetown Science Center laboratory for tissue or DNA samples to allow standardization with REA

microsatellite DNA allele scoring were not fulfilled. Microsatellite DNA amplification was conducted under laminar flow and consisted of 100–200 ng of genomic DNA, 1× polymerase chain reaction (PCR) buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM forward (fluorescently labelled) and reverse primer, and 0.1 U *Taq* DNA polymerase (Promega) in 10 μL. Amplifications were carried out on either a PTC-200 or PTC-225 thermal cycler (MJ Research) using the following: initial denaturing at 94 °C for 2 min, 35 cycles of 94 °C for 40 s, 56 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Fragment analysis was performed on an Applied Biosystems' ABI 3100 Genetic Analyser, as described in King *et al*. (2001). GENESCAN 2.1 Analysis software and GENOTYPER 3.6 fragment analysis software (Applied Biosystems) were used to score, bin, and output allelic (and genotypic) data.

Mitochondrial DNA amplification and sequencing

Two regions of the mtDNA genome were amplified and sequenced. A region of the noncoding control region (CR) was amplified by double-stranded PCR using primers L15926 (5′-TCAAAGCTTACACCAGTCTTGTAAACC-3′) and H16498 (5′-CCTGAACTAGGAACCAGATG-3′) (Kocher *et al*. 1989; Shields & Kocher 1991) for all *Z. hudsonius*, except the KUNHM specimens used to validate the REA data. The CR sequence of interest could not be amplified for these specimens using the primers LI5320 and ZAP5PLr as described in REA. In order to amplify the CR fragment of interest several internal primers were developed: DLIF1 (5′-TTTACCATTATCCATTCATGCTT-3′), DLIF2 (5′-CAGCACCCAAAGCTGATATT-3′), DLIR1 (5′-TTAAGCCTGACTGAATGTGG-3′). Ultimately a pairing of the primers DLIF1 and H16498 were able to amplify a portion of the CR approximately 366 bp in length. *Z. princeps* mtDNA ($N = 7$) was amplified using primers L15398 (5′-ATCAGCACCCAAAGCTGATATTC-3′) (REA) and H16498. PCRs consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C (denaturation), 1 min at 48 °C (annealing), and 2 min at 72 °C (extension), concluding with a final extension period of 5 min at 72 °C. The KUNHM specimens were amplified using an annealing temperature of 55 °C. The mitochondrial cytochrome *b* (cyt *b*) gene was amplified by double-stranded PCR using two primers designed for this study: L14398A (5′-CCAATGACATGAAAAATCATCG-3′) and H15634A (5′-TGGTTTACAAGACCAGAGTAA-3′). PCRs consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing), and 2 min at 72 °C (extension), concluding with a final extension period of 10 min at 72 °C. Polymerase chain reactions consisted of 25 μL total volume, containing 2.5 μL of $MgCl_2$-free buffer, 2.5 μL of $MgCl_2$ solution, 0.5 μL of dNTPs (2.5 mM each), 1.25 μL of each primer (10 μM), 1 U *Taq* polymerase, three μL of template (*c*. 50–100 ng double-stranded DNA), and 13.75 μL of sterile water. Negative controls, which did not include template DNA, were set up alongside PCRs as checks for contamination of PCR reagents. PCR products were purified with exonuclease I and shrimp alkaline phosphatase. The CR and cyt *b* PCR products were sequenced using ABI BigDye version 3.1 terminator cycle sequencing chemistry, with sequences read by an ABI 3100 Genetic Analyser (Applied Biosystems). The CR fragment was sequenced using primers L15926 or L15398 and H16498 for most specimens. The KUNHM specimens were sequenced using the internal primers DLIF1, DLIF2, DLIR1 and H16498. The cyt *b* gene was sequenced using primers L14398A and H15634A, and when necessary, internal primers for the cyt *b* gene. Internal primers designed specifically for this study were: CytbIF1 (5′-CCATTCCATATATTGGCTCA-3′), CytbIF2 (5′-TCCCATTCCATCCTTACTACA-3′), CytbIR1 (5′-CCAATATATGGAATGGCTGA-3′) and CytbIR2 (5′-GGGGTATTTAATGGGTTTGC-3′). Cycle sequencing reactions consisted of 30 cycles of 20 s at 96 °C (denaturation), 20 s at 50 °C (annealing), and 4 min at 60 °C (extension). Forward and reverse sequences for each individual were assembled using SEQUENCHER 4.5 (Gene Codes), were aligned to a reference sequence from GenBank (*Z. h. preblei*, AY598282 for CR and *Z. trinotatus*, AF119262 for cyt *b*), and were double-checked by two researchers. CLUSTAL X (Thompson *et al*. 1994) was used to obtain multiple sequence alignments for CR and cyt *b*. Alignments of both data sets were performed with default settings and were straightforward as only three sites had indels in the CR data set and no indels were encountered in cyt *b*, as would be expected for this protein-coding gene. Sequences generated in this study are available in GenBank (Accession nos CR DQ664546–DQ664900; cyt *b* DQ664901–DQ665221).

Data analysis

Microsatellite DNA. Observed genotype frequencies were tested for consistency with Hardy–Weinberg and linkage equilibrium expectations using randomization tests implemented by GENEPOP 3.1 (Raymond & Rousset 1995). The Hardy–Weinberg test used the Markov chain randomization test of Guo & Thompson (1992) to estimate exact two-tailed *P*-values for each locus in each sample. Tests for linkage equilibrium were conducted using the randomization method of Raymond & Rousset (1995) for all pairs of loci. Bonferroni adjustments (Rice 1989) determined statistical significance for these and all other simultaneous tests. Average observed (H_O) and expected (H_E) heterozygosities were calculated by BIOSYS-1 (Swofford & Selander 1981). Amount of allelic diversity (expressed as allelic richness; El Mousadik & Petit 1996), estimates of population

subdivision (F_{ST}; Weir & Cockerham 1984), and inbreeding coefficients (F_{IS}) were determined using FSTAT (Goudet 1995). All pairwise F_{ST} estimates between subspecies ($N = 5$) and other intraspecific groupings were tested for significance (i.e. difference from zero) by adjusted permutations using FSTAT. Pairwise R_{ST} values among subspecies were calculated using GENEPOP 3.1 and are provided for comparison with F_{ST} values. F_{ST} assumes allelic diversity results from migration and gene drift, while R_{ST} also measures mutational differences between alleles. The statistical significance of genetic differences between each pair of collections, clusters, and subspecies was tested using the genic differentiation randomization test in GENEPOP. Results were combined over loci using Fisher's method (Sokal & Rohlf 1994).

Several techniques were used to describe genetic relationships between subspecies and collections. We used the model-based clustering method of the program STRUCTURE (Pritchard *et al*. 2000) to infer population structure among collections and probabilistically assign all individuals to detected clusters (k). Due to complex migration patterns assumed to exist among disjunct subspecies, a sequential method of inferring k was used by first identifying the 'uppermost' hierarchical level of population structure followed by subsequent analysis of each cluster to identify within-cluster structure (Evanno *et al*. 2005). In the initial phase, $k = 1$ to $k = 15$ clusters were considered for the 14 collections using a burn-in of 15 000 followed by 100 000 iterations, and 100 independent runs for each k. The optimum number of clusters in the initial phase was identified using Δk as described by Evanno *et al*. (2005). Subsequent analysis of each cluster tested $k = 1$ to $k = C + 3$ (the number of collections (C) included in the subset plus three), with a burn-in of 10 000 followed by 10 000 iterations, and 20 runs for each k. In the within-cluster analyses, k was also determined using the Evanno *et al*. (2005) method. Individual assignment success to the cluster or subcluster of origin was recorded as the highest likelihood of assignment (q) and the percentage of individuals in a cluster with $q \geq 0.90$ (Pritchard *et al*. 2000).

Genetic distances between each pair of collections and subspecies were summarized with D_a (Nei *et al*. 1983), calculated by DISPAN (Institute of Molecular Evolutionary Genetics, The Pennsylvania State University). Simulation has shown that the geometric-based D_a exhibits a stronger linear relationship over shorter divergence time, and therefore, estimates better tree topology than other commonly used genetic distances when analysing microsatellite DNA variation (Takezaki & Nei 1996). An unrooted phylogenetic tree was fitted using the D_a distance matrix and neighbour-joining (NJ) algorithm. TreeView (Page 1996) was used to visualize the tree. The strength of support for each node in the tree was tested by bootstrapping over loci using NJBPOP (J.-M. Cornuet, INRA).

Analysis of molecular variance (AMOVA) was used to partition genetic variation among clusters and subspecies (Excoffier *et al*. 1992). ARLEQUIN 2.0 (Schneider *et al*. 2000) was used to quantify and test statistical significance of observed differentiation between subspecies, between clusters of collections, and within subspecies and clusters.

Mitochondrial DNA. Unique haplotypes for each data partition (CR, cyt *b*) were determined using the program COLLAPSE 1.2 (http://darwin.uvigo.es/software/collapse.html). Sequences of each representative CR haplotype from this study were aligned with the REA data from GenBank and the sequence data from the 15 KUNHM specimens to verify their sequences. CLUSTAL X (Thompson *et al*. 1994) was used to align the CR data set with default settings as only three sites had indels. Haplotypes were then compared using the program COLLAPSE 1.2 (http://darwin.uvigo.es/software/collapse.html) to identify matching haplotypes. Sequences from the 15 KUNHM specimens were not used in any subsequent analyses. To examine whether differences in phylogenetic signal existed between the two mitochondrial data partitions, incongruence length differences (ILD, Farris *et al*. 1994) were calculated in PAUP* by the partition-homogeneity test. Settings for ILD tests were as in parsimony analysis, with uninformative sites excluded, and 1000 replicates per run. The combined data set (cyt *b* and control region) was examined using maximum parsimony (MP) using PAUP* 4.0b10 (Swofford 2002). Parsimony analyses consisted of heuristic searches on unweighted, parsimony-informative (PI) characters with starting trees obtained via stepwise addition, 100 random additions of sequences per run, and tree-bisection–reconnection (TBR) branch swapping on best trees. Nodal support on parsimony trees was assessed using 1000 bootstrap replicates (Felsenstein 1985) with full heuristic searches and Bremer support (Bremer 1988, 1994) using TREEROT version 2b (Sorenson 1999). *Z. princeps* was used as the outgroup in all analyses.

Partitioned Bayesian phylogenetic analysis of the combined data set was conducted using the program MRBAYES 3.0 (Huelsenbeck & Ronquist 2001). The data was partitioned into the control region and first, second, and third codon positions of the cyt *b* gene. The appropriate model of evolution for each partition was chosen with the Akaike information criterion (AIC) implemented by the program MRMODELTEST. A neighbour-joining tree for the combined data set was generated using the JC model and used for the analysis of each partition with MRMODELTEST. Bayesian phylogenetic analyses were run for 1.0×10^7 generations with random starting trees, default priors, four Markov chains and sampling every 1000 generations. Stationarity of the MCMC analyses was determined by plotting –ln L values and individual parameter estimates against generation times. Trees from the burn-in were discarded and the remaining trees used to

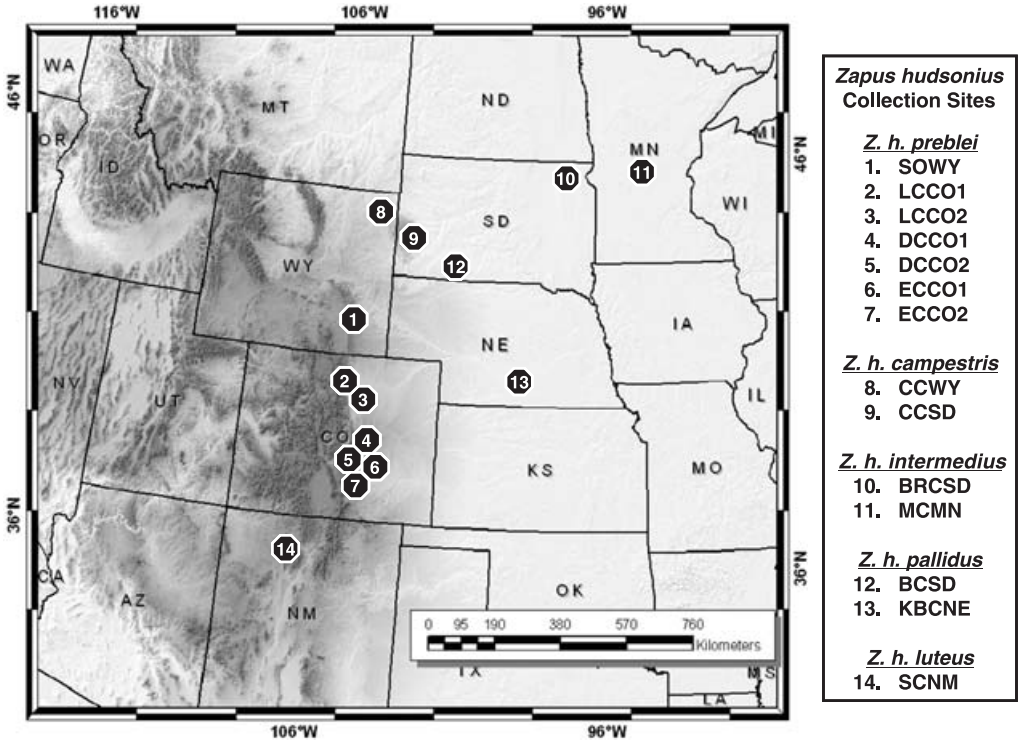

Fig. 1 Generalized collection sites ($N = 14$) representative of five nominal subspecies of *Zapus hudsonius* utilized in this study. See table 1 for collect site name.

calculate posterior probabilities for clades from their frequencies. Two separate analyses were performed and the resulting topologies and posterior probabilities compared.

We investigated intraspecific gene genealogies for the control region and cyt *b* data separately using the haplotype networking approach in the TCS computer program (Clement *et al*. 2000). This analysis implemented the statistical parsimony approach of Templeton *et al*. (1992) and Crandall *et al*. (1994).

Nucleotide diversity of the CR, cyt *b* and combined data sets and exact tests for subspecies and cluster (as defined by STRUCTURE) haplotype differentiation were performed pairwise with 1000 replications in ARLEQUIN 2.0. Total haplotypic variation was partitioned into 'among vs. within' *Z. hudsonius* subspecies using AMOVA. Subspecies- and cluster-level differentiation was assessed using ARLEQUIN 2.0. All AMOVA analyses were conducted in two stages to assess divergence from different evolutionary processes. The first analysis incorporated sequence divergence between haplotypes as well as their frequencies (Φ_{ST}) by calculating either Kimura 2-parameter (CR) or Tamura–Nei (Tamura & Nei 1993) (cyt *b*) estimates. The second analysis, which treated all haplotypes as equally differentiated (i.e. distance = 1.0), assessed the variance distribution based on haplotype frequencies alone (F_{ST}). Differences between haplotype frequencies are assumed to be due to genetic drift. The significance of pairwise Φ_{ST} and F_{ST} values were calculated by permuting haplotypes between populations 1000 times.

Results

Microsatellite DNA

Genotypes at 21 microsatellite DNA loci were determined for 348 *Zapus hudsonius* from 14 locations representing five neighbouring subspecies (see Table 1 for listing and abbreviations; Fig. 1). A high level of genetic diversity was detected among the 14 collections; 280 alleles were observed across 21 loci ranging from 7 at *Zhu*C120 and *Zhu*C130 to 30 at Z.7 (Appendix C). The mean number of alleles per locus was 13.3. Allelic richness estimates for subspecies ranged from 4.6 (*Z. h. luteus*) to 9.4 (*Z. h. intermedius* and *Z. h. pallidus*) (Table 1). Observed heterozygosity was on average 11.5% lower in *Z. h. preblei* than in other subspecies. Observed mean heterozygosity ranged from 53.9% in *Z. h. preblei* to 75.2% in *Z. h. pallidus*. The number of unique alleles observed ranged from 5 (*Z. h. preblei*) to 33 (*Z. h. pallidus*). Estimates of individual pairwise genetic distances, using the proportion of shared alleles, indicated that levels of genetic diversity observed among the 21 microsatellite loci were sufficient to produce unique multilocus genotypes (i.e. genetic distances > zero) for all animals surveyed (distances not presented).

When organized at the collection level, randomization tests showed that genotypes for the majority of locus-by-collection comparisons were consistent with Hardy–Weinberg expectations. A total of 294 locus-by-collection comparisons were made of which 10 (3.4%) were statistically significant after Bonferroni adjustment for multiple tests at overall $\alpha = 0.05$ ($P < 0.0036$). These comparisons consisted of six collections at six loci: CCWY at *Z*.26; BRCSD and MCMN at *Zhu*C119; MCMN, BCSD, and KBCNE at *Zhu*C130; BCSD and KBCNE at *Ztri*19; MCMN at *Zhu*C104; and KBCNE at *Z*.7 (all but the last occurrence due to heterozygote deficiencies). This level of deviation is likely due to one or a combination of factors including substructuring of the sample (i.e. Wahlund effect), inbreeding, or the presence of null alleles. Interestingly, none of the deviations was observed in collections of *Z. h. preblei*, the subspecies targeted for development of 17 of 21 markers surveyed. This observation, combined with the heterozygote deficiencies observed, suggests that differentiation between *Z. h. preblei* and the neighbouring subspecies may have increased the likelihood that null alleles would be present in the nontarget subspecies. The number of deviations from Hardy–Weinberg expectations increased ($N = 26$; 24.8%) when collections were pooled as putative subspecies. The majority ($N = 15$) of the significant deviations (overall $\alpha = 0.05$; $P < 0.01$) were observed among the collections pooled as *Z. h. preblei*. All deviations were the result of heterozygote deficiencies. This result suggests that the allele frequencies of the populations pooled to form this subspecies (*Z. h. preblei*-North and *Z. h. preblei*-South) have achieved different equilibria and that substructuring (i.e. Wahlund effect) has been detected.

Minimal linkage disequilibrium was observed as five of 2700 (0.2%) comparisons of each locus pair across all collections was found to be significant after correction for multiple tests (overall $\alpha = 0.05$, $P < 0.0002$). Linkage disequilibrium was observed between *Z*.20 and *Zhu*D122, *Z*.20 and *Ztri*24, *Zhu*D107 and *Zhu*D122, *Zhu*C12 and *Zhu*C120, and *Z*.48 and *Ztri*2 with the five occurrences distributed among four collections. These findings were likely the result of sampling error, year-class mixing, population mixing, or a combination of the three rather than physical linkage among loci.

Allele frequency heterogeneity was observed throughout the study area. Among 1866 single-locus pairwise tests of allele frequency heterogeneity, 1382 (74.1%) indicated departures from homogeneity after correction for multiple tests ($\alpha = 0.05$, $P < 0.0006$). When testing allele frequency heterogeneity across 21-locus genotypes, highly significant heterogeneity was observed in all 91 pairs of collections ($\alpha = 0.05$, $P < 0.001$).

Results from the STRUCTURE analysis identified that $k = 3$ was the appropriate number of clusters to be recognized at the uppermost hierarchical level among the 14 collections of *Z. hudsonius* genotyped at 21 microsatellite DNA loci. Ten of the 20 independent runs at $k = 3$ resulted in the assignment of the 14 collections to the following clusters: cluster [A] — SOWY, LCCO1, LCCO2, DCCO1, DCCO2, ECCO1, ECCO2 (*Z. h. preblei*); cluster [B] — CCWY, CCSD (*Z. h. campestris*) and BRCSD and MCMN (*Z. h. intermedius*); and cluster [C] — BCSD and KBCNE (*Z. h. pallidus*) and SCNM (*Z. h. luteus*) (Fig. 2). The other 10 independent runs at $k = 3$ resulted in the same clustering with the exception that 27 of 28 *Z. h. preblei* individuals from collection SOWY were assigned to cluster [B], the *Z. h. intermedius*/*Z. h. campestris* cluster. An additional 100 independent STRUCTURE runs were conducted at $k = 3$, with 57 of the runs assigning all of the SOWY animals to the *Z. h. preblei* cluster [A]. All of the 57 runs that assigned the SOWY mice to the *Z. h. preblei* cluster [A] did so with higher average q values (0.93) than the runs that assigned the SOWY animals to the *Z. h. intermedius*/*Z. h. campestris* cluster [B] (average $q = 0.85$). Given the higher number of runs assigning the SOWY collection animals to the *Z. h. preblei* and the higher assignment values of these runs, this clustering was considered the most appropriate. The three clusters detected as the uppermost hierarchical level of population structure suggest the collections constituting *Z. h. preblei* form a distinct grouping from the *Z. h. campestris* and *Z. h. intermedius* collections, which are also distinct from the *Z. h. pallidus* and *Z. h. luteus* collections. This structure was confirmed by 100% correct assignment of each mouse to the cluster-of-origin based on q-values (Table 2). The average value of qMAX for the clusters ranged from 0.96 ([B]) to 0.99 ([C]). Subsequent analysis of the three clusters suggested a strong pattern of genetic differentiation throughout the study area and the presence of $k = 6$ definable subclusters (Fig. 2). This analysis identified phylogeographic discontinuities present within *Z. h. preblei* that corresponded to the northern and southern collections (subclusters 1 and 2, respectively), as well as among clusters allied with *Z. h. campestris* (subcluster 3), *Z. h. intermedius* (subcluster 4), *Z. h. pallidus* (subcluster 5), and *Z. h. luteus* (subcluster 6). A subsequent analysis of the clusters that assigned SOWY animals to cluster [B] resulted in $k = 7$, where the clustering was the same, with the exception being that the SOWY animals were assigned to a unique subcluster. Given these results, we determined that the optimal number of subclusters for these data is six. The strength of the differentiation among these six subclusters was also evident upon inspection of individual assignment results and the average value of qMAX (Table 2). When each individual was assigned to subcluster based on the largest value of q, average assignment success to subcluster of origin was 99.4% (346 of 348 individuals correctly assigned). Two *Z. h. preblei* individuals from *Z. h. preblei*-North (subcluster 1) were incorrectly assigned to *Z. h. preblei*-South (subcluster 2). Average values of qMAX for the six subclusters ranged from 0.94 (*Z. h. preblei–North*)

Table 2 Percentage of *Zapus hudsonius* individuals correctly assigned to one of $k = 3$ initial clusters (A–C) and $k = 6$ subsequent clusters (subclusters) identified by the program STRUCTURE from a survey of 14 collections of mice representing five geographically proximal subspecies surveyed at 21 microsatellite loci. Individuals were assigned to cluster or subcluster based on the largest value of q. Average qMAX and percentage of *Zapus hudsonius* individuals correctly assigned to one of $k = 2$ subclusters identified by the program STRUCTURE based on the $q > 0.90$ criterion are also provided

		Initial cluster ($k = 3$)				Cluster	[A]		[B]		[C]		Subspecies/		Percentage
Subspecies	Collection	[A]	[B]	[C]	Mean qMAX	subcluster ($k = 6$)	1	2	3	4	5	6	cluster designation	Mean qMAX	assigned at $q > 0.90$
Z. h. preblei													*Z. h. preblei*	0.97	100.0% (170)
	SOWY	1.00				1	1.00								
	LCCO1	1.00				1	0.93	0.07							
	LCCO2	1.00				1	0.94	0.06					North	0.94	89.7% (52/58)
					0.98										
	DCCO1	1.00				2		1.00							
	DCCO2	1.00				2		1.00							
	ECCO1	1.00				2		1.00							
	ECCO2	1.00				2		1.00					South	0.98	98.2% (110/112)
Z. h. campestris													*Z. h. campestris*	0.98	95.1% (58/61)
	CCWY		1.00			3			1.00						
	CCSD		1.00		0.96	3			1.00						
Z. h. intermedius													*Z. h. intermedius*	0.99	100.0% (49)
	BRCSD		1.00			4				1.00					
	MCMN		1.00			4				1.00					
Z. h. pallidus															
													Z. h. pallidus	0.99	100.0% (48)
	BCSD			1.00		5					1.00				
	KBCNE			1.00	0.99	5					1.00				
Z. h. luteus													*Z. h. luteus*	0.99	100.0% (20)
	SCNM			1.00		6						1.00			

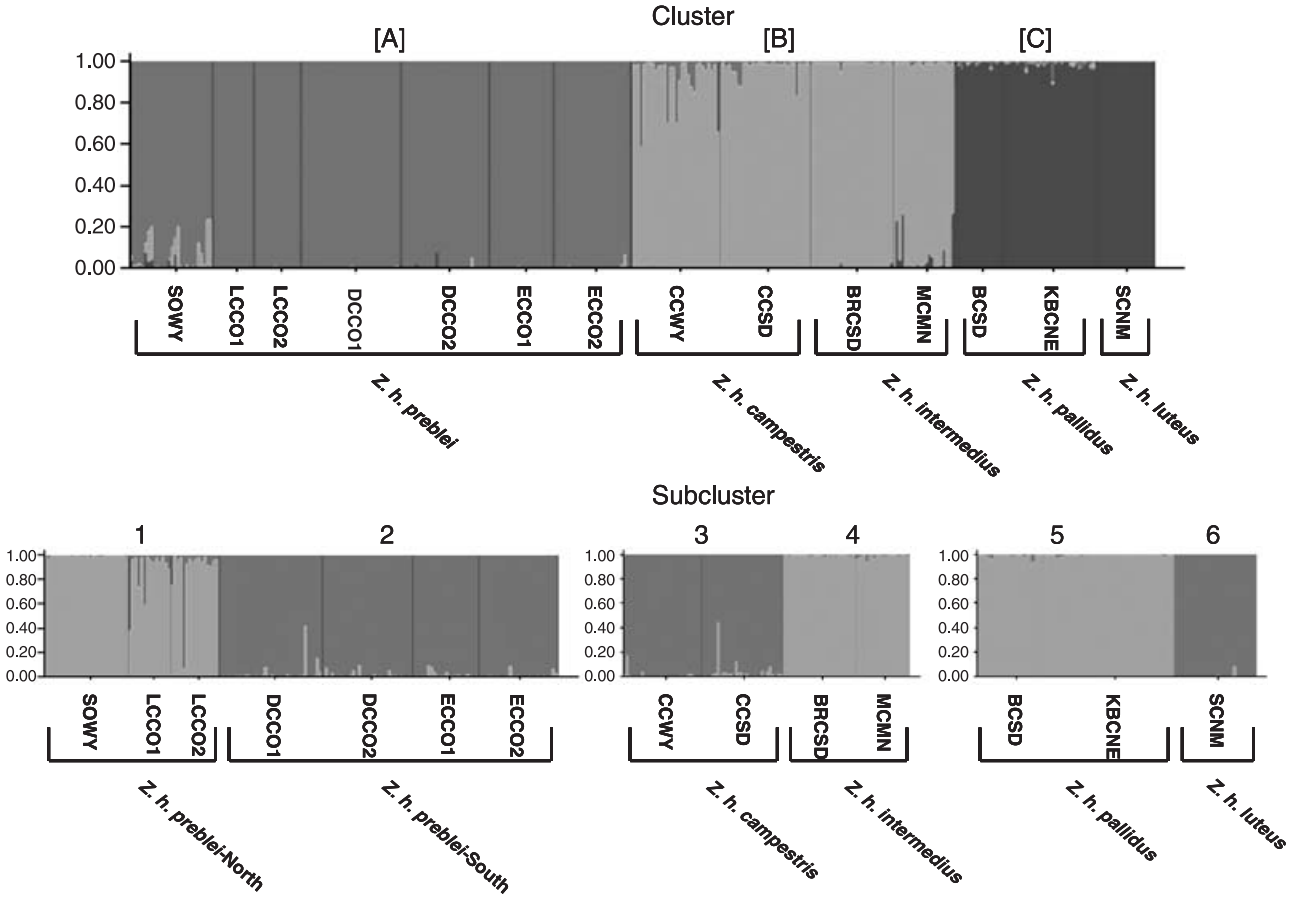

Fig. 2 Summary plots of *q* estimates generated by the sequential cluster analysis of the program STRUCTURE performed on the multilocus ($N = 21$) genotypes of 14 collections of *Zapus hudsonius*. The number of inferred clusters (k) in the initial (uppermost hierarchical level) analysis was three (clusters [A–C]). Each initial cluster was subsequently analysed for within-cluster structure. The sequential analysis further subdivided each cluster into two subclusters for a total of six clusters (1–6). Each individual is represented by a single vertical line, broken into k coloured segments, the length of which is proportional to the membership fraction in each of the k clusters. Individuals are grouped by populations and subspecies as indicated by brackets.

to 0.99 (*Z. h. intermedius*, subcluster 4, *Z. h. pallidus*, subcluster 5; *Z. h. luteus*, subcluster 6). When using $q = 90$ as an assignment threshold, the percentage of correct assignment to subcluster ranged from 89.7% (*Z. h. preblei*–North) to 100.0% (*Z. h. intermedius*, *Z. h. pallidus*, and *Z. h. luteus*). When compared at the subspecies level ($q = 90$ criterion), assignment success ranged from 95.1% (*Z. h. campestris*) to 100% (*Z. h. preblei*, *Z. h. intermedius*, *Z. h. pallidus*, and *Z. h. luteus*).

Pairwise genetic distance values (D_a, Nei *et al*. 1983) were calculated between all collections to investigate evolutionary relationships in allele frequencies. The greatest genetic distances occurred between the *Z. h. luteus* collection and all other collections; the lowest genetic distances were observed between collections from the same subspecies or cluster (as defined by STRUCTURE). The underlying genetic structure of the D_a matrix is illustrated with an unrooted neighbour-joining (NJ) tree (Fig. 3). The patterns observed illustrate high levels of differentiation among and within the five subspecies and reflects the patterns of genetic variation identified by STRUCTURE. The distinctiveness of the *Z. h. preblei* collections was confirmed relative to other subspecies by 98% bootstrap support for separation of the subspecies from all other collections. The SOWY collection from the northernmost portion of the subspecies' range was intermediate between the other northern *Z. h. preblei* collections and the disjunct (by approximately 150 km) *Z. h. campestris* collections. In addition, this figure depicts the differentiation observed: (i) among each of the five subspecies; (ii) within each subspecies (excluding *Z. h. luteus*); and (iii) between two major groupings (*Z. h. preblei*, subclusters 1 and 2; *Z. h. campestris*, subcluster 3; *Z. h. intermedius*, subcluster 4) and (*Z. h. pallidus*, subcluster 5; *Z. h. luteus*, subcluser 6) (100% bootstrap support). The closest genetic relationship among subspecies exists between *Z. h. campestris* and *Z. h. intermedius*.

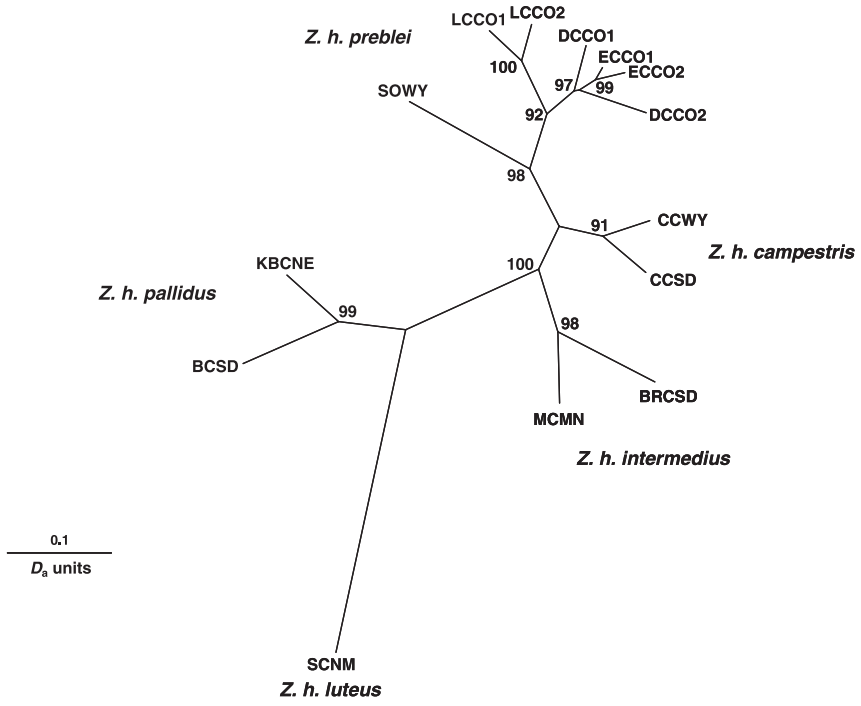

Fig. 3 Unrooted neighbour-joining tree generated from pairwise genetic distance (D_a; Nei *et al*. 1983) values between all collections of *Zapus hudsonius* generated from multilocus microsatellite genotypes. Branch lengths are proportional to D_a units. Numbers along branches represent bootstrap support for nodes.

Table 3 F_{ST} values (below the diagonal) generated from a survey of 21 microsatellite loci in five geographically proximal subspecies of *Zapus hudsonius*. All F_{ST} estimates were statistically significant from zero ($\alpha = 0.05$, $P < 0.001$) after 1000 permutations. R_{ST} values (above the diagonal) are provided for comparison with F_{ST}

Subspecies	*Z. h. preblei*	*Z. h. campestris*	*Z. h. intermedius*	*Z. h. pallidus*	*Z. h. luteus*
Z. h. preblei		0.1561	0.4442	0.6259	0.7708
Z. h. campestris	0.1063		0.2075	0.4759	0.6474
Z. h. intermedius	0.1810	0.1069		0.3455	0.4910
Z. h. pallidus	0.2146	0.1812	0.1555		0.1816
Z. h. luteus	0.3250	0.3032	0.2714	0.1832	

A high level of genetic differentiation was also observed in F_{ST} comparisons at the subcluster (data not presented) and subspecies (Table 3) scale. All 24 tests of significance in pairwise F_{ST} values (clusters and subspecies) were statistically greater than zero ($P < 0.001$) indicating numerous significant genetic discontinuities throughout this portion of *Z. hudsonius*' range. The lowest pairwise F_{ST} value was observed between the two *Z. h. preblei* subclusters (North and South, 0.10). F_{ST} estimates were highest between the *Z. h. luteus* subcluster and the *Z. h. prebei*, *Z. h. campestris*, and *Z. h. intermedius* subclusters (all values ≥ 0.27). Moderately high F_{ST} estimates were observed between the *Z. h. preblei* and *Z. h. campestris* subclusters (average 0.12), and between *Z. h. pallidus* and the *Z. h. intermedius* and *Z. h. campestris* subclusters (average 0.15). F_{ST} estimates at the subspecies level (all collections and subclusters pooled) mirrored those observed among subclusters, with the highest estimates observed between *Z. h. preblei* and *Z. h. luteus*, *Z. h. campestris* and *Z. h. luteus*, and *Z. h. intermedius* and *Z. h. luteus* (Table 3). The lowest F_{ST} estimates were observed among *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius* averaging 0.13. R_{ST} values were on average 2.1 times larger than corresponding F_{ST} values and ranged from 0.16 (*Z. h. preblei*–*Z. h. campestris*) to 0.78 (*Z. h. preblei*–*Z. h. luteus*) (Table 3). The ratio of R_{ST} to F_{ST} values ranged between 1.0 (*Z. h. luteus*–*Z. h. pallidus*) and 2.9 (*Z. h. preblei*–*Z. h. pallidus*). The observed R_{ST} values suggest that the differentiation observed among most of the subspecies is considerable as mutational processes have acted to increase differentiation over that observed through random genetic drift.

Quantitative estimates of hierarchical gene diversity (AMOVA) among subspecies and subclusters also identified statistically significant genetic structuring. A comparison between the five subspecies (all collections pooled) determined that 18.4% ($P < 0.001$) of the genetic variation occurred between subspecies and 81.6% ($P < 0.001$) was

due to differentiation within subspecies. Further partitioning resulted in 14.8% ($P < 0.001$) of the variation being distributed between subspecies, 8.6% ($P < 0.001$) observed among collections within subspecies, and 76.6% ($P < 0.001$) of the variance detected within collections. A comparison between the six subclusters (all collections pooled) identified by STRUCTURE yielded results identical to those observed for subspecies partitions –18.4% ($P < 0.001$) of the variance due to differences between subclusters and 81.6% ($P < 0.001$) was attributable to differentiation within subclusters. Partitioning variation among collections within subclusters resulted in slightly more variation being distributed among subclusters (15.4%, $P < 0.001$), less variation (6.7%; $P < 0.001$) among collections within subclusters, and more variation (77.9%; $P < 0.001$) attributed to variation in collections within subclusters than was observed for subspecies. When subclusters were grouped into subspecies, 11.9% ($P < 0.024$) of the variation was distributed between subspecies, 7.8% ($P < 0.001$) was between subclusters within subspecies, and 80.3% ($P < 0.001$) of the variance was observed within clusters.

Mitochondrial DNA

Sequence data from *Z. hudsonius* individuals from 14 collections representing five neighbouring subspecies were analysed for sequence variation at two mitochondrial genes, the 5′-end of the CR (374 bp, 332 individuals) and cyt *b* gene (1006 bp, 320 individuals). In the CR data set 25 haplotypes were recovered with 28 (7.5%) sites variable, 27 (7.2%) sites parsimony-informative, and three indels parsimony-informative when coded as a fifth base (Appendix D). For the cyt *b* region, 56 haplotypes were recovered with 116 (11.5%) sites variable, 84 (8.3%) sites parsimony-informative and no indels present (Appendix E).

Control region sequences were obtained for 15 of the KUNHM specimens utilized by REA. When these sequences were compared to the REA data from GenBank and representative CR haplotypes from this study, 13 of the sequences were different than those reported by REA (Table B.1, Appendix B). All seven of the *Z. h. campestris* that were reported as having *Z. h. preblei* haplotypes by REA were found to have common *Z. h. campestris* haplotypes. Given these results, the validity of all the REA data from the mitochondrial DNA genome is called into question, and therefore was not combined with the data from this study for any analyses.

Adjusted (net) mtDNA sequence divergence estimates (Kimura 2-parameter, CR and Tamura–Nei, cyt *b*) between *Z. hudsonius* subspecies were relatively low, averaging 3.37% (CR) and 4.43% cyt *b* (not shown). Net distances between *Z. hudsonius* subspecies ranged between 0.29% (CR) and 0.18% (cyt *b*) for the most closely related subspecies, *Z. h. campestris* and *Z. h. intermedius*, to 5.63% (*Z. h. campestris*–*Z. h. luteus*, CR) and 7.11% (*Z. h. campestris*–*Z. h. pallidus*, cyt *b*). *Z. h. preblei* was least divergent from *Z. h. intermedius* (0.57%, CR and 0.68%, cyt *b*), and most divergent from *Z. h. luteus* (5.08%, CR) or *Z. h. pallidus* (6.76%, cyt *b*). Divergence estimated from CR between *Z. h. preblei* and *Z. h. campestris* (1.03%) was nearly twice that observed between *Z. h. preblei* and *Z. h. intermedius*. Estimates of divergence between *Z. hudsonius* subspecies and *Z. princeps*, used as an outgroup in phylogenetic analyses, were higher (10.33%, CR and 19.87%, cyt *b*).

Nucleotide diversity within subspecies was low across the study, and ranged from 0.0000 in *Z. h. luteus* (both gene regions) to 0.0060 and 0.0030 in *Z. h. pallidus* (Table 4, CR and cyt *b*, respectively). Twenty-five (CR) and 56 (cyt *b*) haplotypes were observed study-wide for *Z. hudsonius*, yet none were shared among the five subspecies (Appendices B and C). For *Z. h. preblei*, 1 of 4 CR haplotypes and none of the 21 cyt *b* haplotypes were shared between the northern and southern clusters.

Statistical parsimony analyses of sequence data from each mitochondrial gene region produced largely concordant haplotype networks. In the CR analysis with gaps included as a fifth character state, the 0.95 limit for connections was eight steps and in the cyt *b* data set the 0.95 limit was 13 steps. Both analyses produced multiple networks, but both included a single network including all *Z. h. preblei*, *Z. h. intermedius*, and *Z. h. campestris* haplotypes. In the CR analysis, a second network was formed by *Z. h. pallidus* and *Z. h. luteus* haplotypes (figure not shown), where these haplotypes formed two separate networks corresponding to subspecies based on cyt *b* (Fig. 4). Within the (*Z. h. preblei*–*intermedius*–*campestris*) networks, haplotypes made up of individuals from each subspecies clustered together (subspecies outlined in boxes) and were separated from other subspecies' haplotypes by one (CR) to six (cyt *b*, *preblei*-*intermedius*, Fig. 4) mutational steps. The same three *Z. h. intermedius* individuals had CR (ZhiCR_E) and cyt *b* (ZhiCB_A, Fig. 4) haplotypes that had fewer mutational steps to a *Z. h. campestris* haplotype than to other *Z. h. intermedius* haplotypes; yet these haplotypes were not found in any *Z. h. campestris* individuals ($N = 61$).

Analysis of molecular variance (AMOVA) of data from each mitochondrial gene region indicated the presence of strong, significant genetic differentiation among the five *Z. hudsonius* subspecies surveyed. Global Φ_{ST} (0.89 for CR and 0.96 for cyt *b*) and F_{ST} (0.36 for CR and 0.29 for cyt *b*; not shown) values were high and significantly different from zero ($P < 0.00001$). As no haplotypes were shared among the five *Z. hudsonius* subspecies, F_{ST} values (a measure of haplotype frequencies only) generated by the AMOVA analysis have limited biological resolution and are discussed only for comparison to previous findings (REA). A global Φ_{ST} of 0.96 indicates that nearly all of the haplotype variance (96%) was distributed between subspecies, a result

Table 4 Sequence diversity at the mitochondrial DNA control (372 base pairs) and cytochrome *b* (1006 base pairs) regions observed in five neighbouring subspecies of *Zapus hudsonius*

	Control region				Cytochrome *b*				Combined			
Taxon	N	Polymorphic sites	Haplotypes	Nucleotide diversity	N	Polymorphic sites	Haplotypes	Nucleotide diversity	N	Polymorphic sites	Haplotypes	Nucleotide diversity
Z. h. preblei	160	3	4	0.0030	146	16	21	0.0013	133	19	25	0.0017
Z. h. intermedius	47	9	9	0.0041	47	22	13	0.0028	45	31	17	0.0032
Z. h. campestris	61	5	5	0.0025	60	13	13	0.0010	59	18	15	0.0013
Z. h. pallidus	47	9	5	0.0060	48	13	8	0.0030	47	22	9	0.0038
Z. h. luteus	19	1	2	0.0000	19	0	1	0.0000	18	1	2	0.0000

consistent with the absence of any shared haplotypes observed between subspecies. In general, comparisons among all subspecies resulted in higher Φ_{ST} values for cyt *b* than for CR but with one exception, the comparison between *Z. h. intermedius* and *Z. h. campestris*, which also were the lowest Φ_{ST} estimates (CR = 0.59, cyt *b* = 0.53). A comparison of haplotype variation among the three subspecies proposed for synonymy by REA, *Z. h. preblei*, *Z. h. intermedius* and *Z. h. campestris*, resulted in 73% (CR) and 83% (cyt *b*) of the variance being distributed among subspecies. Moreover, AMOVA results suggest that *Z. h. preblei* is distinct from other neighbouring subspecies and is evolutionarily more similar to *Z. h. intermedius* than to *Z. h. campestris*, as Φ_{ST} estimates were lowest with *Z. h. intermedius* (Φ_{ST} = 0.67, CR and Φ_{ST} = 0.82, cyt *b*) and slightly higher when compared with *Z. h. campestris* (Φ_{ST} = 0.79, CR and Φ_{ST} = 0.89, cyt *b*). This finding differs from the pattern observed during analysis of the nuclear DNA, which found *Z. h. preblei* to exhibit a lower genetic distance between *Z. h. campestris* than *Z. h. intermedius*. All estimates of subspecies from different mitochondrial clades (Fig. 5A) were above 90%, with both *Z. h. preblei* and *Z. h. campestris* reaching 98% in comparisons with *Z. h. luteus*. Haplotype variation distributed among the clusters indicated by the nuclear microsatellite loci by the program STRUCTURE was similar to that observed among the subspecies, as 90.0% of the CR variation and 95.4% of the cyt *b* variation ($P < 0.001$) was attributable to differentiation among subclusters and the remainder due to variation within. These findings mirrored the high level of genetic differentiation observed upon survey of these subclusters with the nuclear microsatellite DNA markers. Exact tests for differentiation based on haplotype frequencies for both gene regions and clusters ($N = 15$ comparisons) were statistically significant ($P < 0.001$).

Combined mitochondrial DNA

Results of ILD tests were not significant ($P = 0.23$), plus phylogenetic analyses of the individual data partitions were congruent with respect to the major clades recovered (data not shown); so data was combined for individuals occurring in both data sets (303 individuals), then collapsed into 69 unique haplotypes (68 *Zapus hudsonius*, 1 *Z. princeps* Table 4). The strict consensus tree of 8032 most parsimonious trees (Fig. 5a) revealed two well-supported clades within *Z. hudsonius*. One clade, supported by 100% of bootstrap pseudoreplicates and a decay index of 17, was comprised of *Z. h. pallidus* and *Z. h. luteus* haplotypes, and the two *Z. h. luteus* haplotypes formed a well-supported monophyletic group. The other major clade recovered was comprised of *Z. h. intermedius*, *Z. h. campestris*, and *Z. h. preblei* haplotypes (bootstrap = 100, decay index 36, Fig. 5a). Within this clade, none of the *Z. hudsonius* subspecies were recovered as

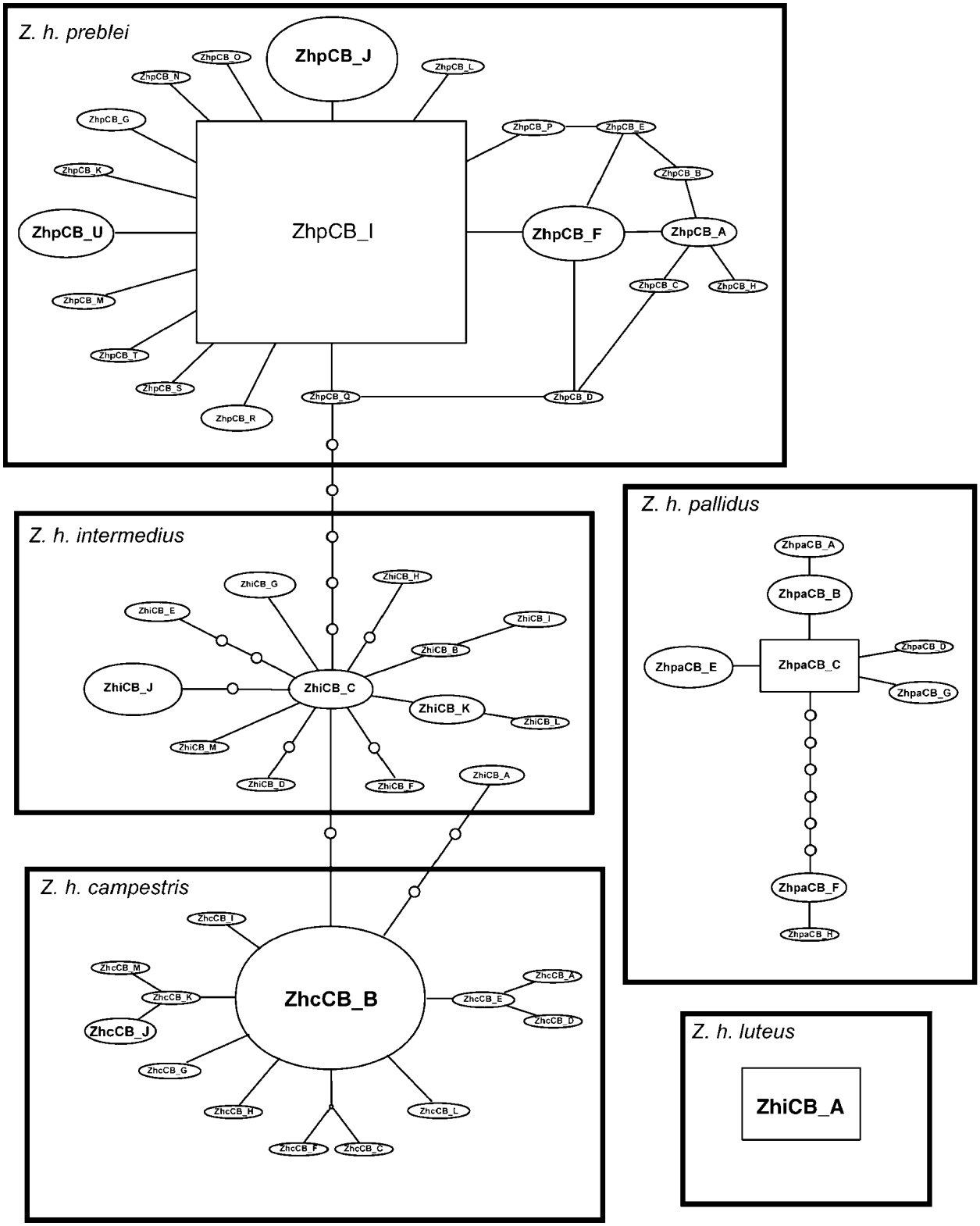

Fig. 4 *Zapus hudsonius* haplotype networks resulting from the statistical parsimony analysis of the cyt *b* data set. The set of haplotypes belonging to each subspecies of *Z. hudsonius* are outlined by boxes. No haplotypes were shared among subspecies. Haplotypes identified as the root of each network by the program TCS (Clement *et al*. 2000) are represented by a square and all other haplotypes are represented as circles. The size of each haplotype symbol is proportional to the number of copies observed in the data set. Haplotypes for *Z. h. pallidus* and *Z. h. luteus* are not connected to the network containing *Z. h. preblei* due to the differentiation exceeding the 95% limit for connections for cyt *b* (13%).

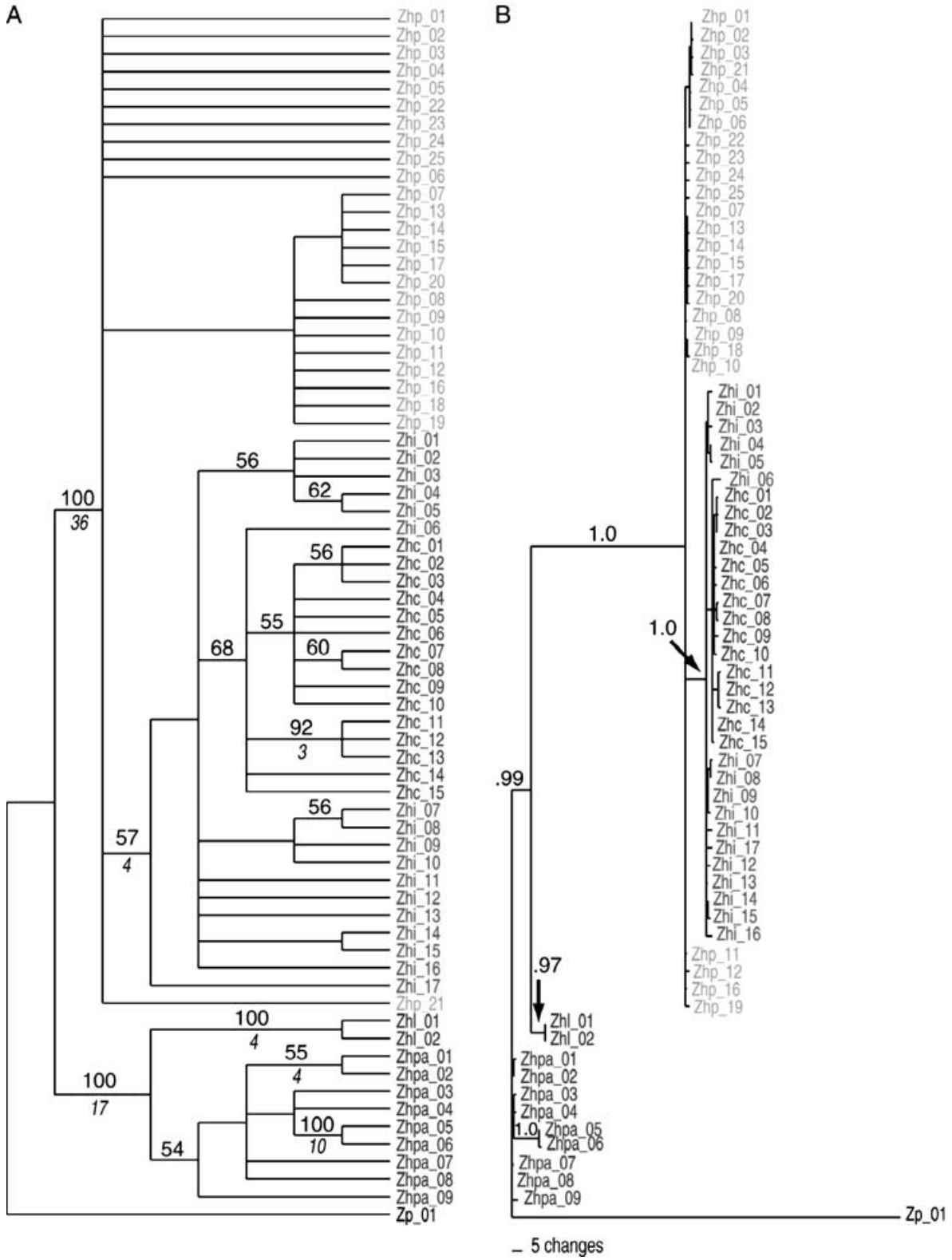

Fig. 5 Phylogenetic hypotheses for five *Zapus hudsonius* subspecies based upon combined mitochondrial DNA data: (A) strict consensus of 8032 most parsimonious trees (359 steps, CI = 0.8273) and (B) phylogram resulting from partitioned Bayesian analysis. Numbers above branches in A are bootstrap proportions and italicized numbers below branches in A are decay indices, numbers above branches in B are posterior probabilities of nodes.

monophyletic, yet the *Z.h. campestris/intermedius* haplotypes formed a clade paraphyletic to *Z. h. preblei* haplotypes (bootstrap values of 57, decay index of 4, Fig. 5a). Generally, nodes within this major clade were poorly supported by bootstrap and decay indices.

Models select using the AIC by MRMODELTEST for the control region and first, second, and third codon positions of the cyt *b* gene were HKY+I+G, K80+I, GTR+I, and GTR respectively. In all Bayesian analyses stationarity was reached by 50 000 generations, therefore the first 50 trees were discarded as burn-in and the remaining trees used to calculate topology and posterior probabilities. Topologies of the consensus trees from the two Bayesian analyses were identical and posterior probabilities for each clade varied by no more than 0.01 (Fig. 5b, one of two Bayesian consensus trees). The trees resulting from the Bayesian analysis were similar in topology to the MP consensus (Fig. 5a), with the major difference being the relationship between *Z. h. pallidus* and *Z. h. luteus* — they are not sister taxa in the Bayesian analysis (Fig. 5b). In the phylogram of the Bayesian tree (Fig. 5b), it is notable that branch lengths were short within the *Z. h. campestris/intermedius/preblei* clade, with the longest branch (and the only one with a significant posterior probability) separating *Z. h. preblei* from *Z. h. campestris/intermedius* haplotypes.

Discussion

The disjunct collections represented in the present study may not qualify as distinct species because their reproductive barrier is extrinsic, but their apparently long-standing separation has resulted in the accretion of considerable genetic differentiation that is reflected in geographically congruent patterns of divergence at multiple DNA markers. The magnitude of the observed differentiation was considerable and supported by highly significant findings for nearly every statistical comparison, regardless of the genome or the taxa under consideration. Given the strong structuring of nuclear multilocus genotypes, the presence of only subspecies-specific mtDNA haplotypes, and that the genetic discontinuities correspond with previously proposed taxonomic classifications based on geographic separation and morphological variation among these *Zapus hudsonius* subspecies (Krutzsch 1954; Hafner *et al*. 1981), we find no genetic evidence warranting taxonomic revision among the *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius* as previously proposed (REA), nor suggesting the need to alter *Z. h. preblei*'s standing under the ESA. Moreover, the level of differentiation observed between *Z. h. preblei* inhabiting southern Wyoming and Larimer County, CO (*Z. h. preblei*-North) and mice sampled from Douglas and El Paso counties, CO (*Z. h. preblei*-South) indicates that this subspecies is comprised of at least two genetically distinct populations worthy of individual management consideration. Spatial genetic structuring apparent in *Z. hudsonius* subspecies is reminiscent of that observed in *Zapus trinotatus* (Pacific jumping mouse). Through a landscape genetics approach, Vignieri (2005) explored the interactions between dispersal and environmental characteristics and found that both restricted dispersal and habitat-directed movement were likely forces shaping fine-scale genetic patterns between populations. Sufficient data have also been presented to suggest that the differentiation observed between the *Z. h. pallidus* and *Z. h. luteus* clade and other *Z. hudsonius* subspecies warrants further study and serious consideration given to species-level recognition for the former.

The findings of this study are contradictory in nearly every comparison with the conclusions drawn from the only other molecular genetics data available on the taxonomic status of *Z. hudsonius* subspecies (REA). It is imperative that the disparities between the previous and present study be discussed due to the conservation implications of synonymizing *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius* as proposed by REA, and the misleading precedent that may be accepted for evaluating the genetic basis for listing under the ESA unless these discrepancies are understood by scientists and properly portrayed to resource managers. The largest differences between the two studies dealt with the presence or absence of haplotype sharing among subspecies and the philosophical perspective with which the researchers interpreted the findings (see Conservation implications). It was particularly troubling that the CR sequences of 13 specimens reported by REA to possess shared haplotypes between subspecies were not substantiated when sequenced for this study (see Appendix B). Equally troubling was that 80 of the 222 total sequences deposited to GenBank by REA cannot be directly linked to the specimens listed in Appendix 2 of REA because accession numbers are not provided and the isolate names listed on GenBank for the 80 sequences do not match ID numbers given in REA. The inability to directly link the sequences deposited in GenBank to the specimens listed in REA and the associated locality data renders the data useless. Possible reasons for the different sequences reported by REA are contamination, mislabelling of samples, or other procedural incongruity. Given that all the specimens re-evaluated in the current study were found by REA to have haplotypes that were shared among subspecies, the disagreement in DNA sequences reported for these individuals calls into question all of the results of REA based on the mitochondrial DNA genome and prevents meaningful analysis of the combined data. Moreover, this study and that of REA differ in sampling regime, number of molecular characters sequenced and microsatellite fragments surveyed, test statistics applied to AMOVAS, methods used to portray genealogical relationships, and criteria used to determine uniqueness. We will discuss the nature of these

discrepancies and suggest how each approach impacts the conclusions drawn regarding subspecies distinctness.

An appropriate sampling strategy is central to the successful delineation of population genetic and phylogeographic structures (Baverstock & Moritz 1996). The 'population-oriented' strategy used in the present study differed significantly from the 'one or few individuals per site across a broad geographical area' approach applied by REA. This study focused on collections ranging from 14 to 33 individuals from recognized geographic populations, increasing the likelihood that the majority of haplotypes within a population would be surveyed. Total genetic variation within *Z. hudsonius* can be hierarchically partitioned: (i) between subspecies, (ii) among populations within subspecies, and (iii) within populations. The sampling strategy and subsequent analyses performed by REA likely underestimated the level of within-population variation, effectively inflating within-subspecies variance, while potentially lowering the total variance attributed to between-subspecies differentiation. This sampling strategy may have decreased the likelihood that distinctiveness (as defined by REA) would be demonstrated. While both sampling strategies have their strengths and weaknesses and a combination of the two may ultimately be preferred, we believe the strategy used in the present study is the more pragmatic approach for testing statistical significance under the hypothesis testing approach espoused by REA, in which uniqueness was based on partitioning of variation within and between subspecies. The systemic error identified in the REA CR region sequences prevented an analysis of the combined data.

Large numbers of microsatellite DNA loci provide better estimates of population genetic parameters given the stochastic variation (including size homoplasy) expected among independent loci (Takezaki & Nei 1996; Adams *et al*. 2004). Given that microsatellite loci are densely interspersed in eukaryotic genomes (Katti *et al*. 2001), five polymorphic microsatellite DNA loci may not provide sufficient representation of the nuclear DNA variation present (Beaumont & Nichols 1996; Pritchard & Rosenberg 1999) or provide a sufficiently robust multilocus genotype for population/phylogeographic comparisons (Smouse & Chevillon 1998). In a test to determine the number of diagnosable clusters present in their microsatellite DNA data set using STRUCTURE, REA found the variability of likelihood estimates to be high, and concluded that the eight clusters identified by the analysis were poorly defined. This result is consistent with the findings of Pritchard *et al*. (2000) in which simulations found five loci were insufficient to resolve clusters. The 21 loci surveyed in the present study allowed identification of both deep and shallower levels of population structure, consequently resulting in six definable clusters. The present study also generated larger estimates of subspecies differentiation (F_{ST}, 0.11–0.34; R_{ST}, 0.17–0.78) than found in the previous study (F_{ST}, 0.01–0.16), which is consistent with the AMOVA that found a higher percentage of variation was distributed between subspecies (17.4%) than reported by REA (7.5–8.9%). The increased coverage of the nuclear genome afforded by the larger number of microsatellite loci yielded increased resolution of the phylogeographic structure present in *Z. hudsonius*. R_{ST} differentiation observed among most of the subspecies is considerable, not of recent occurrence, and not simply the result of genetic drift.

The more than twofold differential in the estimate of variation between subspecies provided by Φ_{ST} (this study) and F_{ST} (REA) broaches important issues regarding the conclusions drawn from the respective studies and underscores the fundamental differences between the two test statistics (Φ_{ST} and F_{ST}) generated by AMOVA. Φ_{ST} incorporates sequence divergence between haplotypes (providing the option of several distance metrics), as well as determining haplotype frequencies. Evolutionary differences among the haplotypes (i.e. mutations) are incorporated into calculations of the test statistics. F_{ST}, which utilizes conventional F-statistics, treats all haplotypes, regardless of their evolutionary interrelationships, as equally differentiated (i.e. distance = 1.0), and assesses variance distribution based on haplotype frequencies alone. Differences between haplotype frequencies are assumed to be due to genetic drift. Given that no haplotypes were shared among the five *Z. hudsonius* subspecies in the present study, Φ_{ST} would appear to be the most appropriate statistic as F_{ST} values (a measure of haplotype frequencies only) have limited biological resolution or significance in this particular situation. When Φ_{ST} and F_{ST} values are similar in magnitude, any population differentiation is likely due to genetic drift, but when Φ_{ST} is large (as is the case for all subspecies comparisons in this study), regardless of the F_{ST}, evolutionary depth in separations among haplotypes is implied. Moreover, if REA's critical test of uniqueness for *Z. h. preblei* mtDNA sequence data were applied (i.e. greater molecular variance be demonstrated between subspecies than within), the results obtained between all subspecies compared in the present study exceed this uniqueness test criterion (all Φ_{ST} values were > 0.50). By opting to utilize F_{ST} and failing to recognize the evolutionary differences among observed haplotypes, we contend that REA have utilized an inappropriate criterion for determining subspecies uniqueness.

For microsatellite DNA comparisons, REA's critical test of uniqueness for subspecies and historic genetic exchangeability was twofold: most variation was observed between subspecies in pairwise AMOVA comparisons (i.e. $F_{ST} \geq 0.50$) and multiple private alleles are found at higher frequency than shared alleles at a majority of loci. These criteria often are not met among accepted species due to constraints on fixation indices generated from

microsatellite DNA loci created by high heterozygosity levels and homoplasy (Hedrick 1999; Balloux *et al*. 2000). Although several R_{ST} values observed among the five subspecies compared in this study approached or exceeded the proposed criterion (Table 3), we contend the proposed criteria for microsatellite markers are unfounded because of the longer coalescent times associated with the nuclear genome (four times longer than mitochondrial DNA), and they likely have little biological meaning because a fundamental evolutionary process (e.g. mutation) is ignored.

In phylogeographic studies, principles of genealogical concordance have become a conceptual framework for identifying deeper units within a species (Ball & Avise 1992; Avise 2000). Data presented here for *Z. hudsonius* subspecies are genealogically concordant in three out of four levels defined by Avise (2000): concordance between DNA characters (I); concordance between genomes (II); and concordance of gene tree partitions with independent biogeographic and systematic information (IV). First, there was concordance between the patterns of change in DNA sequences from the two mtDNA regions surveyed, seen in the similar, subspecies-specific patterns apparent in analyses of each gene region (Appendices D and E; Fig. 4) and from the fact that branch support for several nodes distinguishing subspecies increases, and becomes significant, when data sets are combined (Fig. 5a). The addition of more characters (e.g. cyt *b* and CR) shows that there is more 'depth' to the divergence between *Z. h. preblei*, *Z. h. campestris* and *Z. h. intermedius* (i.e. greater number of inferred substitutions separating haplotypes and greater genetic distances) than examination of fewer bases of mtDNA revealed. Fine-scale patterns also become apparent, such as genetic structuring between northern and southern *Z. h. preblei* populations. Because intraspecific phylogeographic data sets are predisposed to have fewer informative characters and relationships between haplotypes are unlikely to be hierarchical in nature, traditional phylogenetic methods can lead to inadequately portrayed genealogical relationships (Brower *et al*. 1996). The haplotype network analysis adopted here depicted ancestral relationships and the nonrandom distribution of mutations among lineages, which resulted in the diagnostic haplotypic structure observed between subspecies and not observed in the more traditional parsimony analyses performed in this study or the distance-based approach utilized by REA. When forced into a phylogenetic comparison (e.g. parsimony analysis), *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius* exhibited shallow gene genealogies (at both CR and cyt *b*) — an intraspecific pattern consistent with ancestral polymorphism and incomplete lineage sorting; patterns that would be expected among recently diverged groups (Maddison 1997; Arbogast *et al*. 2002) and not uncommonly seen within rodent species (e.g. *Peromyscus boylii*, Tiemann-Boege *et al*. 2000; *Neotoma floridana*, Edwards & Bradley 2001; *Microtus agrestis*, Jaarola & Searle 2002). For the reason that evolution occurs at varying rates depending primarily on environmental- and demographic-based dynamics, we contend that sequence variation/divergence thresholds are inappropriate for intra or interspecific taxonomic designations. Alternatively, as genetic variation is apportioned among and within taxa, decisions on taxonomic recognition should be rendered on a case-by-case basis. Lastly, concordant patterns observed in the two mtDNA genes and nuclear microsatellites (Figs 2 and 3) are in agreement with the disjunct geographic distributions and morphological differences that led to the subspecies descriptions (Krutzsch 1954; Hafner *et al*. 1981) and lends confidence that actual evolutionary relationships have been recovered.

Conservation implications

Criteria chosen for delineating isolated intraspecific groupings (e.g. subspecies, phylogroups, distinct population segments, evolutionary significant units, designatable units, or management units) become of critical importance when the entity's continued existence is at risk (Moritz 1994b; Paetkau 1999). Given that the issue at hand constitutes an intraspecific comparison, we are concerned about the methodological pitfalls of applying approaches based on species-level inference. Mayr (1942) defined a subspecies as 'a geographically localized subdivision of the species, which differs genetically and taxonomically from other subdivisions of the species.' While this definition infers that subspecies have unique ranges and are diagnosable (Patten & Unitt 2002), they are not necessarily reproductively isolated or they would qualify as species. Systematists have taken diverse positions on how 'taxonomically' may be interpreted. Some have asserted that this term infers long-term reproductive isolation and that the various components should be monophyletic (see Zink 2004 and references therein). Recently diverged intraspecific taxa can be characterized by multiple ancestral lineages at the time of divergence and might not exhibit reciprocal monophyly (Hudson & Coyne 2002; Funk & Omland 2003; Green 2005). We contend that since adaptation, diagnosability and biological speciation may exist well before achieving reproductive isolation and monophyly, these latter criteria have no basis under which to become standards for subspecies designation.

Similarly, genetic distinctness criteria that are inflexible represent a conservation stratagem that could impact the potential for future evolutionary change within the intraspecific unit of management and may lead to extirpation or extinction of discrete and evolutionarily significant intraspecific diversity. We found Ramey *et al*.'s (2005) core genetic-based criteria for *Zapus hudsonius* subspecies distinctness (i.e. greater variation between subspecies than within) to be (i) too stringent to account for relatively

recently radiated taxa, (ii) scientifically unfounded as this criterion would not distinguish certain well-established species (Forbes *et al*. 1995; see Vignieri *et al*. 2006 and references therein), (iii) unachievable under some circumstances with the test statistics applied (F_{ST}; Hedrick 1999), and (iv) untenable given that important evolutionary processes (e.g. mutation) are disregarded. Consequently, these criteria should not be considered as an accepted standard when addressing ESA actions or for international conservation laws. Alternatively, we recommend application of the requirements for describing subspecies under the ESA proposed by Haig *et al*. (in press), which state that all such designations should meet the 'discreteness' and 'significance' criteria defined for listing a distinct population segment (DPS) under the Act.

The level of discontinuity observed among the subspecies in this study should not be considered minor or 'shallow' (Avise 2004) simply because the level of DNA sequence differentiation is relatively low, and not accompanied by the presence of reciprocal monophyly, demonstrable phenotypic divergence (but see Vignieri *et al*. 2006), or obvious adaptive significance. The differential magnitude observed between Φ_{ST} (distance and frequency) and F_{ST} (frequency) values for both mtDNA CR and cyt *b* suggests moderate micro-evolutionary depth among the five subspecies accompanied by phylogeographical structuring of haplotypes and multilocus genotypes within *Z. h. preblei*. The diagnostic nature of the haplotype variation between *Z. h. preblei* and other subspecies (there are no credible published data suggesting otherwise) and large degree of nuclear genetic differentiation at microsatellite loci suggests that these subspecies (and certain geographic populations within) have been reproductively isolated for such time that they appear to be on independent evolutionary trajectories, while having nearly achieved complete lineage sorting. We have identified strongly differentiated units of conservation (which could qualify as DPSs) that are entirely consistent with current taxonomy and support protection of the threatened population segment under the ESA. We conclude the subspecies surveyed in this study do not warrant synonymy, as proposed for *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius*; rather, they constitute distinct evolutionary lineages that merit separate management consideration, and those populations facing demographic challenges (e.g. *Z. h. preblei*-North, *Z. h. preblei*-South) should be afforded high conservation priority. The strong concurrence among patterns of mitochondrial and nuclear DNA variation observed in this study suggests that formal recognition of the relationships revealed will assist in preserving the potential for future evolutionary change within and among these subspecies.

A detailed comparison similar to that performed here for all subspecies of *Z. hudsonius* is warranted. Such a study would likely shed light on the significance of the considerable differentiation observed among the *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius* clade and will allow differentiation observed for *Z. h. pallidus* and *Z. h. luteus* to be observed in the best available context.

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Tim King's research emphasis involves the application of molecular genetic markers to the phylogenetics, phylogeography, population structure, and captive breeding management of declining species. This contribution is yet another effort to assist natural resource managers with guidance in identifying appropriate units of conservation. John Switzer is working in King's laboratory with research interests that include systematics, phylogeography and conservation genetics. Cheryl Morrison is working in King's laboratory with research interests in the fields of evolutionary and conservation genetics, including molecular systematics and taxonomy issues, phylogeography, and population genetics. Mike Eackles, Colleen Young, and Barbara Lubinski are biologists that specialize in DNA fragment and sequence analyses in King's laboratory. Paul Cryan's research focuses on the ecology, behavior, and biogeography of at-risk species of mammals.

Appendix A

Listing of *Zapus hudsonius* museum specimens used in the present study including the museum identifier, the USGS individual identifier, the abbreviated collection name (Table 1), the county and state each specimen was collected, and the subspecies designation. Tissues of specimens from three museums were utilized: Denver Museum of Nature and Science, Denver, Colorado; University of Kansas, Natural History Museum, Lawrence, Kansas, and the University of New Mexico, Museum of Southwestern Biology, Albuquerque, New Mexico

Museum identifier	Individual identifier	Study collection	County	State	Designated subspecies
Denver Museum of Nature and Science					
CHG9901	CHG9901	SOWY	Laramie	Wyoming	*preblei*
CTA9802	CTA9802	SOWY	Albany	Wyoming	*preblei*
CTN9901	CTN9901	SOWY	Albany	Wyoming	*preblei*
DOU9901	DOU9901	SOWY	Converse	Wyoming	*preblei*
DUC9901	DUC9901	SOWY	Albany	Wyoming	*preblei*
ELB9901	ELB9901	SOWY	Laramie	Wyoming	*preblei*
ELB9902	ELB9902	SOWY	Laramie	Wyoming	*preblei*
EPB9901	EPB9901	SOWY	Laramie	Wyoming	*preblei*
FRC9802	FRC9802	SOWY	Albany	Wyoming	*preblei*
LUM9901	LUM9901	SOWY	Platte	Wyoming	*preblei*
LUM9902	LUM9902	SOWY	Platte	Wyoming	*preblei*
LUM9903	LUM9903	SOWY	Platte	Wyoming	*preblei*
MCW9901	MCW9901	SOWY	Albany	Wyoming	*preblei*
MCW9902	MCW9902	SOWY	Albany	Wyoming	*preblei*
NLR9901	NLR9901	SOWY	Albany	Wyoming	*preblei*
NSB9901	NSB9901	SOWY	Laramie	Wyoming	*preblei*
RBC9901	RBC9901	SOWY	Platte	Wyoming	*preblei*
SBC9901	SBC9901	SOWY	Laramie	Wyoming	*preblei*
SSC9901	SSC9901	SOWY	Albany	Wyoming	*preblei*
SSC9902	SSC9902	SOWY	Albany	Wyoming	*preblei*
SSC9903	SSC9903	SOWY	Albany	Wyoming	*preblei*
SYB9901	SYB9901	SOWY	Platte	Wyoming	*preblei*
YCA9806	YCA9806	SOWY	Laramie	Wyoming	*preblei*
YCA9807	YCA9807	SOWY	Laramie	Wyoming	*preblei*
YCB9801	YCB9801	SOWY	Laramie	Wyoming	*preblei*
YCB9802	YCB9802	SOWY	Laramie	Wyoming	*preblei*
YCB9803	YCB9803	SOWY	Laramie	Wyoming	*preblei*
YCB9804	YCB9804	SOWY	Laramie	Wyoming	*preblei*
CER-9801	CER-9801	LCCO1	Larimer	Colorado	*preblei*
CER-9802	CER-9802	LCCO1	Larimer	Colorado	*preblei*
CER-9803	CER-9803	LCCO1	Larimer	Colorado	*preblei*
CER-9804	CER-9804	LCCO1	Larimer	Colorado	*preblei*
CER-9805	CER-9805	LCCO1	Larimer	Colorado	*preblei*
CER-9806	CER-9806	LCCO1	Larimer	Colorado	*preblei*
HRK-9801	HRK-9801	LCCO1	Larimer	Colorado	*preblei*
HRK-9802	HRK-9802	LCCO1	Larimer	Colorado	*preblei*
HRK-9803	HRK-9803	LCCO1	Larimer	Colorado	*preblei*
HRK-9804	HRK-9804	LCCO1	Larimer	Colorado	*preblei*
CP9806, random # 205	CP-205	LCCO1	Larimer	Colorado	*preblei*
MC-9801	MC-9801	LCCO1	Larimer	Colorado	*preblei*
MC-9803	MC-9803	LCCO1	Larimer	Colorado	*preblei*
NFP-9801	NFP-9801	LCCO1	Larimer	Colorado	*preblei*
NFP-9802	NFP-9802	LCCO1	Larimer	Colorado	*preblei*
BG-9801	BG-9801	LCCO2	Larimer	Colorado	*preblei*
BG-9802	BG-9802	LCCO2	Larimer	Colorado	*preblei*
PGC-9801	PGC-9801	LCCO2	Larimer	Colorado	*preblei*
SP9803, random # 125	SP-125	LCCO2	Larimer	Colorado	*preblei*
SP9802, random # 169	SP-169	LCCO2	Larimer	Colorado	*preblei*
SP9805, Random # 170	SP-170	LCCO2	Larimer	Colorado	*preblei*
SP9807, Random # 223	SP-223	LCCO2	Larimer	Colorado	*preblei*

Appendix A *Continued*

Museum identifier	Individual identifier	Study collection	County	State	Designated subspecies
SP9804, Random # 243	SP-243	LCCO2	Larimer	Colorado	*preblei*
SP9806, Random # 336	SP-336	LCCO2	Larimer	Colorado	*preblei*
SP9812, Random # 367	SP-367	LCCO2	Larimer	Colorado	*preblei*
SP9810, Random # 375	SP-375	LCCO2	Larimer	Colorado	*preblei*
SP9811, Random # 674	SP-674	LCCO2	Larimer	Colorado	*preblei*
SP9801, Random # 746	SP-746	LCCO2	Larimer	Colorado	*preblei*
SP9809, Random # 861	SP-861	LCCO2	Larimer	Colorado	*preblei*
YG-9801	YG-9801	LCCO2	Larimer	Colorado	*preblei*
YG-9803	YG-9803	LCCO2	Larimer	Colorado	*preblei*
MA98133, Random # 127	MAY-127	DCCO1	Douglas	Colorado	*preblei*
MA9809, Random # 165	MAY-165	DCCO1	Douglas	Colorado	*preblei*
MA98122, Random # 215	MAY-215	DCCO1	Douglas	Colorado	*preblei*
MA98201, Random # 229	MAY-229	DCCO1	Douglas	Colorado	*preblei*
MA98135, Random # 234	MAY-234	DCCO1	Douglas	Colorado	*preblei*
MA9801, Random # 254	MAY-254	DCCO1	Douglas	Colorado	*preblei*
MA98104, Random # 268	MAY-268	DCCO1	Douglas	Colorado	*preblei*
MA98108, Random # 281	MAY-281	DCCO1	Douglas	Colorado	*preblei*
MA98134, Random # 368	MAY-368	DCCO1	Douglas	Colorado	*preblei*
MA98132, Random # 374	MAY-374	DCCO1	Douglas	Colorado	*preblei*
MA9802, Random # 385	MAY-385	DCCO1	Douglas	Colorado	*preblei*
MA98136, Random # 408	MAY-408	DCCO1	Douglas	Colorado	*preblei*
MA98120, Random # 416	MAY-416	DCCO1	Douglas	Colorado	*preblei*
MA98130, Random # 429	MAY-429	DCCO1	Douglas	Colorado	*preblei*
MA98138, Random # 452	MAY-452	DCCO1	Douglas	Colorado	*preblei*
MA98131, Random # 494	MAY-494	DCCO1	Douglas	Colorado	*preblei*
MA98124, Random # 497	MAY-497	DCCO1	Douglas	Colorado	*preblei*
MA98121, Random # 517	MAY-517	DCCO1	Douglas	Colorado	*preblei*
MA98102, Random # 532	MAY-532	DCCO1	Douglas	Colorado	*preblei*
MA98106, Random # 694	MAY-694	DCCO1	Douglas	Colorado	*preblei*
MA98204, Random # 706	MAY-706	DCCO1	Douglas	Colorado	*preblei*
MA98107, Random # 714	MAY-714	DCCO1	Douglas	Colorado	*preblei*
MA98123, Random # 748	MAY-748	DCCO1	Douglas	Colorado	*preblei*
MA9805, Random # 785	MAY-785	DCCO1	Douglas	Colorado	*preblei*
MA9810, Random # 798	MAY-798	DCCO1	Douglas	Colorado	*preblei*
MA98103, Random # 817	MAY-817	DCCO1	Douglas	Colorado	*preblei*
MA98203, Random # 822	MAY-822	DCCO1	Douglas	Colorado	*preblei*
MA9804, Random # 880	MAY-880	DCCO1	Douglas	Colorado	*preblei*
MA9806, Random # 933	MAY-933	DCCO1	Douglas	Colorado	*preblei*
MA98137, Random # 940	MAY-940	DCCO1	Douglas	Colorado	*preblei*
MA98202, Random # 946	MAY-946	DCCO1	Douglas	Colorado	*preblei*
MA9811, Random # 964	MAY-964	DCCO1	Douglas	Colorado	*preblei*
MA9813	MAY-9813	DCCO1	Douglas	Colorado	*preblei*
MA9814	MAY-9814	DCCO1	Douglas	Colorado	*preblei*
WH9801, Random # 715	WH-9801	DCCO2	Douglas	Colorado	*preblei*
WH9802, Random # 911	WH-9802	DCCO2	Douglas	Colorado	*preblei*
WH9803, Random # 629	WH-9803	DCCO2	Douglas	Colorado	*preblei*
WH9804, Random # 675	WH-9804	DCCO2	Douglas	Colorado	*preblei*
WH9805, Random # 849	WH-9805	DCCO2	Douglas	Colorado	*preblei*
WH9806, Random # 961	WH-9806	DCCO2	Douglas	Colorado	*preblei*
WH98100, Random # 573	WH-98100	DCCO2	Douglas	Colorado	*preblei*
WH98101, Random # 789	WH-98101	DCCO2	Douglas	Colorado	*preblei*
WH98102, Random # 672	WH-98102	DCCO2	Douglas	Colorado	*preblei*
WH98103, Random # 884	WH-98103	DCCO2	Douglas	Colorado	*preblei*
WH98104, Random # 719	WH-98104	DCCO2	Douglas	Colorado	*preblei*
WH98105, Random # 635	WH-98105	DCCO2	Douglas	Colorado	*preblei*
WH98106, Random # 603	WH-98106	DCCO2	Douglas	Colorado	*preblei*

Appendix A *Continued*

Museum identifier	Individual identifier	Study collection	County	State	Designated subspecies
WH98107, Random # 716	WH-98107	DCCO2	Douglas	Colorado	*preblei*
WH98108, Random # 208	WH-98108	DCCO2	Douglas	Colorado	*preblei*
WH98109, Random # 958	WH-98109	DCCO2	Douglas	Colorado	*preblei*
WH98110, Random # 543	WH-98110	DCCO2	Douglas	Colorado	*preblei*
WH98120, Random # 768	WH-98120	DCCO2	Douglas	Colorado	*preblei*
WH98121, Random # 113	WH-98121	DCCO2	Douglas	Colorado	*preblei*
WH98300, Random # 140	WH-98300	DCCO2	Douglas	Colorado	*preblei*
WH98301, Random # 204	WH-98301	DCCO2	Douglas	Colorado	*preblei*
WH98302, Random # 814	WH-98302	DCCO2	Douglas	Colorado	*preblei*
WH98303, Random # 610	WH-98303	DCCO2	Douglas	Colorado	*preblei*
WH98304, Random # 190	WH-98304	DCCO2	Douglas	Colorado	*preblei*
WH98305, Random # 314	WH-98305	DCCO2	Douglas	Colorado	*preblei*
WH98306, Random # 612	WH-98306	DCCO2	Douglas	Colorado	*preblei*
WH98309, Random # 120	WH-98309	DCCO2	Douglas	Colorado	*preblei*
WH98311, Random # 860	WH-98311	DCCO2	Douglas	Colorado	*preblei*
WH98312, Random # 883	WH-98312	DCCO2	Douglas	Colorado	*preblei*
WH98313, Random # 924	WH-98313	DCCO2	Douglas	Colorado	*preblei*
MCA9801	Zhp-01	ECCO1	El Paso	Colorado	*preblei*
MCA9805	Zhp-03	ECCO1	El Paso	Colorado	*preblei*
MCA9806	Zhp-04	ECCO1	El Paso	Colorado	*preblei*
98KC01	Zhp-05	ECCO1	El Paso	Colorado	*preblei*
98 PV01	Zhp-06	ECCO1	El Paso	Colorado	*preblei*
98SC01	Zhp-07	ECCO1	El Paso	Colorado	*preblei*
98SC02	Zhp-08	ECCO1	El Paso	Colorado	*preblei*
98SC03	Zhp-09	ECCO1	El Paso	Colorado	*preblei*
98SC04	Zhp-10	ECCO1	El Paso	Colorado	*preblei*
98SC05	Zhp-11	ECCO1	El Paso	Colorado	*preblei*
98SC07	Zhp-13	ECCO1	El Paso	Colorado	*preblei*
DMC01	Zhp-15	ECCO1	El Paso	Colorado	*preblei*
DMC02	Zhp-16	ECCO1	El Paso	Colorado	*preblei*
BS0198	Zhp-17	ECCO1	El Paso	Colorado	*preblei*
BS0298	Zhp-18	ECCO1	El Paso	Colorado	*preblei*
LR0198	Zhp-19	ECCO1	El Paso	Colorado	*preblei*
LR0298	Zhp-20	ECCO1	El Paso	Colorado	*preblei*
LR0398	Zhp-21	ECCO1	El Paso	Colorado	*preblei*
DC9802	Zhp-23	ECCO1	El Paso	Colorado	*preblei*
DC9803	Zhp-24	ECCO1	El Paso	Colorado	*preblei*
DC9804	Zhp-25	ECCO1	El Paso	Colorado	*preblei*
DC9805	Zhp-26	ECCO1	El Paso	Colorado	*preblei*
GCC0198	Zhp-27	ECCO1	El Paso	Colorado	*preblei*
University of Kansas Natural History Museum					
KU109972	Zhc-116	NA	Custer	South Dakota	*campestris*
KU109963	Zhc-124	NA	Lawrence	South Dakota	*campestris*
KU109978	Zhc-117	NA	Custer	South Dakota	*campestris*
KU109984	Zhc-118	NA	Custer	South Dakota	*campestris*
KU109985	Zhc-119	NA	Custer	South Dakota	*campestris*
KU110013	Zhc-115	NA	Custer	South Dakota	*campestris*
KU123592	Zhc-097	NA	Carter	Montana	*campestris*
KU123597	Zhc-095	NA	Carter	Montana	*campestris*
KU112661	Zhc-126	NA	Lawrence	South Dakota	*campestris*
KU112663	Zhc-127	NA	Lawrence	South Dakota	*campestris*
KU115700	Zhi-033	NA	Burleigh	North Dakota	*intermedius*
KU115730	Zhi-037	NA	Walworth	South Dakota	*intermedius*
KU112665	Zhc-128	NA	Lawrence	South Dakota	*campestris*
KU153706	Zhpa-050	NA	Leavenworth	Kansas	*pallidus*
KU110033	Zhpa-051	NA	Bennett	South Dakota	*pallidus*

Appendix A *Continued*

Museum identifier	Individual identifier	Study collection	County	State	Designated subspecies
Museum of Southwestern Biology, University of New Mexico					
None	MSB-41518	MCMN	Morrison	Minnesota	*intermedius*
84916	MSB-41532	MCMN	Morrison	Minnesota	*intermedius*
84917	MSB-41533	MCMN	Morrison	Minnesota	*intermedius*
90860	MSB-80766	MCMN	Morrison	Minnesota	*intermedius*
90861	MSB-80767	MCMN	Morrison	Minnesota	*intermedius*
90862	MSB-80768	MCMN	Morrison	Minnesota	*intermedius*
90863	MSB-80769	MCMN	Morrison	Minnesota	*intermedius*
90864	MSB-80770	MCMN	Morrison	Minnesota	*intermedius*
90865	MSB-80771	MCMN	Morrison	Minnesota	*intermedius*
90866	MSB-80772	MCMN	Morrison	Minnesota	*intermedius*
90867	MSB-80773	MCMN	Morrison	Minnesota	*intermedius*
90868	MSB-80774	MCMN	Morrison	Minnesota	*intermedius*
90869	MSB-80778	MCMN	Morrison	Minnesota	*intermedius*
90870	MSB-80779	MCMN	Morrison	Minnesota	*intermedius*
90871	MSB-80780	MCMN	Morrison	Minnesota	*intermedius*
90872	MSB-80781	MCMN	Morrison	Minnesota	*intermedius*
90873	MSB-80782	MCMN	Morrison	Minnesota	*intermedius*
90874	MSB-80783	MCMN	Morrison	Minnesota	*intermedius*
90875	MSB-80784	MCMN	Morrison	Minnesota	*intermedius*
90876	MSB-80785	MCMN	Morrison	Minnesota	*intermedius*
90943	MSB-80786	MCMN	Morrison	Minnesota	*intermedius*
Museum of Southwestern Biology, University of New Mexico					
56982	MSB-3826	SCNM	Sandoval	New Mexico	*luteus*
56993	MSB-3827	SCNM	Sandoval	New Mexico	*luteus*
56994	MSB-3828	SCNM	Sandoval	New Mexico	*luteus*
56996	MSB-3829	SCNM	Sandoval	New Mexico	*luteus*
56984	MSB-3830	SCNM	Sandoval	New Mexico	*luteus*
56995	MSB-3831	SCNM	Sandoval	New Mexico	*luteus*
56979	MSB-3832	SCNM	Sandoval	New Mexico	*luteus*
56983	MSB-3833	SCNM	Sandoval	New Mexico	*luteus*
56997	MSB-3834	SCNM	Sandoval	New Mexico	*luteus*
56980	MSB-3835	SCNM	Sandoval	New Mexico	*luteus*
None	MSB-3836	SCNM	Sandoval	New Mexico	*luteus*
56981	MSB-3837	SCNM	Sandoval	New Mexico	*luteus*
56985	MSB-3838	SCNM	Sandoval	New Mexico	*luteus*
56990	MSB-3839	SCNM	Sandoval	New Mexico	*luteus*
56991	MSB-3840	SCNM	Sandoval	New Mexico	*luteus*
56992	MSB-3841	SCNM	Sandoval	New Mexico	*luteus*
56986	MSB-3842	SCNM	Sandoval	New Mexico	*luteus*
56989	MSB-3843	SCNM	Sandoval	New Mexico	*luteus*
56987	MSB-3844	SCNM	Sandoval	New Mexico	*luteus*
56988	MSB-3845	SCNM	Sandoval	New Mexico	*luteus*

Appendix B

Validation of Ramey et al. *(2005) control region sequence data*

Ramey *et al.* (2005) (REA) reported 10 haplotypes shared between and among *Zapus hudsonius* subspecies. REA used decades-old, dried museum skins as a principal source of genetic material for selected collections used in the phylogeographic comparison. Given the discrepancy between the results of REA and this study (King *et al.*; KEA) regarding haplotype sharing, we were concerned that this methodological decision may have introduced unnecessary ambiguity to the findings. For example, REA reported the presence of *Z. h. preblei* haplotypes in DNA extracted from five dried museum skins of Z. *h. campestris* collected from Custer County, SD. The authors suggested this finding indicated recent gene flow and alluded to the presence of these haplotypes as a critical element in the decision to recommend synonymy of these subspecies. KEA sampled 31 *Z. h. campestris* recently from the same site in Custer County, South Dakota used by REA, along with 30 additional specimens from neighbouring Crook County, Wyoming. All individuals were subjected to mtDNA CR and cyt *b* sequence analysis. All 61 individuals were determined to posses *Z. h. campestris*-specific mtDNA haplotypes. Moreover, the same conclusion was reached with the microsatellite loci, as no *Z. h. campestris* individual from either of these collections was assigned to *Z. h. preblei*. Given the prominent role the haplotypes obtained for the five museum skins from Custer County, South Dakota and two additional specimens from Carter County, Montana have played in the conclusions drawn by REA and the absence of sharing observed in a large sample by this study, we felt compelled to validate the previous findings.

Here we present the results of attempts to validate the mitochondrial DNA control region (CR) sequences reported by REA for seven *Z. h. campestris* dried museum skin specimens obtained from the Kansas University Natural History Museum (KUNHM) and reported to possess four *Z. h. preblei* haplotypes. In addition, eight other specimens were obtained to assist in validation of four additional shared haplotypes. An alignment of the CR sequences for the 15 KUNHM specimens with the sequences generated for the five *Z. hudsonius* subspecies surveyed in this study (KEA) was performed. From this alignment, the identity of the 15 specimens was readily apparent. The follow description and associated table (Table B.1) are provided to assist the reader in assessing the validity of the mtDNA sequences reported by and conclusions made by REA.

Haplotype C/P1

Eleven specimens were listed by REA as having haplotype C/P1: nine *Z. h. preblei* (DMNH10405, DMNH10258, DMNH10270, DMNH10404, DMNH10406, DMNH10407, DMNH9568, PIONEER9568, PIONEER9B89) and two *Z. h. campestris* (KU109984, KU109985). When the REA GenBank data is compared with this study (KEA), all individuals were found to match the haplotype ZhpCR_A. Tissue samples from two of the specimens (KU109984 and KU109985) were obtained from the KUMNH and sequenced to verify the results of REA. The sequences obtained for the two specimens were not the same as those reported by REA (haplotype C/P1), both specimens had haplotype ZhcCR_D, a haplotype only observed in *Z. h. campestris* by KEA.

Haplotype C/P2

Fourteen specimens were reported by REA to have haplotype C/P2: 12 *Z. h. preblei* (DMNH9579, DMNH9313, DMNH1315, DMNH10380, DMNH9565, DMNH9563, DMNH9566, DMNH9573, DMNH9572, DMNH9571, DMNH9574, DMNH10607) and two *Z. h. campestris* (KU109978, KU123592). When the REA GenBank data is compared to that of KEA, all individuals were found to match the haplotype ZhpCR_B. Tissue samples from two of the specimens (KU109978 and KU123592) were obtained from the KUMNH and sequenced. The sequences obtained for the two specimens were not the same as those reported by REA (haplotype C/P2); both specimens had haplotype ZhcCR_D, a haplotype only observed in *Z. h. campestris* by KEA.

Haplotype C/P3

Twenty-six specimens were listed as having haplotype C/P3 by REA: 24 *Z. h. preblei* and two *Z. h. campestris* (KU110013, KU123597). When the REA GenBank data is compared to that of KEA, all of these individuals were found to match the haplotype ZhpCR_C. Tissue samples from the two *Z. h. campestris* specimens (KU110013 and KU123597) were obtained from the KUMNH and sequenced. The sequences obtained for the two specimens were not the same as those reported by REA (haplotype C/P3); both specimens had haplotype ZhcCR_D, a haplotype only observed in Z. *h. campestris*.

Haplotype C/P4

Ten specimens are listed as having haplotype C/P4 by REA: nine *Z. h. preblei* and one *Z. h. campestris* (KU109972). When the REA GenBank data is compared to that of KEA, all of these individuals were found to match the haplotype ZhpCR_D. A tissue sample from the *Z. h. campestris* specimen (KU109972) was obtained from the KUMNH and sequenced to verify the results of REA. The sequence obtained for the specimen was not the same as that reported by REA (haplotype C/P4); the specimen had haplotype ZhcCR_A, a haplotype only observed in *Z. h. campestris*.

Table B1. Results of 15 museum specimens (Kansas University Museum of Natural History; KUMNH) sequenced to verify selected results from Ramey *et al*. (2005) (REA). Information provided includes: museum catalogue number, collection information, designated subspecies, haplotype reported by REA, corresponding haplotype in King *et al*. (this manuscript; KEA), the haplotype observed after re-analysis, and the results of an assignment to subspecies based on microsatellite DNA analysis for the seven specimens reported to possess *Z. h. preblei* haplotypes by REA. The number of loci used (in parenthesis) and the ratio of likelihood of assignment to the designated subspecies divided by the score for the REA haplotype designation (i.e. *Z. h. preblei*) are provided

Catalogue number	Collection county, state	Designated subspecies	REA haplotype	Corresponding KEA haplotype	Observed haplotype§	Assignment to subspecies
KU123597*	Carter County, MT	*campestris*	C/P3	ZhpCR_C	ZhcCR_D	*campestris* (8) [1.7]
KU123592*	Carter County, MT	*campestris*	C/P2	ZhpCR_B	ZhcCR_D	*campestris* (13) [1.7]
KU110013*	Custer County, SD	*campestris*	C/P3	ZhpCR_C	ZhcCR_D	*campestris* (12) [2.5]
KU109984*	Custer County, SD	*campestris*	C/P1	ZhpCR_A	ZhcCR_D	*campestris* (11) [2.3]
KU109985*	Custer County, SD	*campestris*	C/P1	ZhpCR_A	ZhcCR_D	*campestris* (9) [1.1]
KU109978*	Custer County, SD	*campestris*	C/P2	ZhpCR_B	ZhcCR_D	*campestris* (15) [2.2]
KU109972*	Custer County, SD	*campestris*	C/P4	ZhpCR_D	ZhcCR_A	*campestris* (9) [2.8]
KU112665	Lawrence County, SD	*campestris*	L/PAL/C1	ZhlCR_A	KU45	
KU109963	Lawrence County, SD	*campestris*	L/PAL/C1	ZhlCR_A	ZhcCR_A	
KU110033	Bennett County, SD	*pallidus*	L/PAL/C1	ZhlCR_A	New¶	
KU112661	Lawrence County, SD	*campestris*	L/PAL/C2	none†	ZhcCR_D	
KU153706	Leavenworth County, KS	*pallidus*	L/PAL/C2	none†		
KU112663	Lawrence County, SD	*campestris*	C9/INT-VII	none‡	ZhcCR_D	
KU115730	Walworth County, SD	*intermedius*	C9/INT-VII	none‡	ZhcCR_D	
KU115700	Burleigh County, ND	*intermedius*	C8/10/INT-VI	ZhcCR_D		

MT, Montana; SD, South Dakota; KS, Kansas; ND, North Dakota.
**Z. h. campestris* specimens from KUMNH identified by REA as having *Z. h. preblei* haplotypes.
†Haplotype not observed by King *et al*. (this study); designated as KU45 by REA in GenBank.
‡Haplotype not observed by King *et al*.; designated as KU26 by REA in GenBank.
§Thirteen of 15 KUMNH specimens were observed (this study) to have different haplotypes than those reported by REA.
¶This haplotype is unique as it was not observed by either study.

Table B1 presents the results of the comparison among the seven *Z. hudsonius campestris* specimens. All seven individuals were observed to posses *Z. h. campestris* haplotypes consistent with the KUMNH identification. In addition to the CR comparison, microsatellite DNA was surveyed in these specimens. Multilocus genotypes of the seven individuals was subjected to a maximum-likelihood assignment test to determine whether the specimen was most closely related to the baseline collections of Z. *h. campestris* or *Z. h. preblei* surveyed by KEA following the methods presented in the text. The results of the assignment testing indicated that all seven individuals were on average 2.0 times more likely to be *Z. h. campestris* than *Z. h. preblei*.

Haplotype L/PAL/C1

Haplotype L/PAL/C1 was reported to be shared among three subspecies of *Z. hudsonius* by REA. Eleven specimens were listed as having haplotype L/PAL/C1 by REA: seven *Z. h. luteus* (MSB58370, MSB56980, MSB56986, MSB56987, MSB56991, MSB56993, MSB62096, and NK856), two *Z. h. campestris* (KU112665, KU109963), and one *Z. h. pallidus* (KU110033). Of these 11 specimens only one, NK856, has consistent numbering between Appendix 2 of REA and data accessioned in GenBank that allows for direct comparison. When the REA GenBank data was compared to that of KEA, NK856 was found to match the haplotype ZhlCR_A. Ten other specimens were also found to match this haplotype: six *Z. h. luteus* (MSB20, MSB21, MSB23, MSB24, MSB25, MSB26, MSB27), two *Z. h. campestris* (KU25, KU28), one *Z. h. pallidus* (KU53). Given this evidence, we believe haplotype L/PAL/C1 reported by REA is the same as haplotype ZhlCR_A of KEA. Tissue samples from three of the specimens (KU112665, KU109963 and KU110033) were obtained from KUMNH and sequenced to verify the results of REA. The sequences obtained were not the same as those reported by REA (haplotype L/PAL/C1): specimen KU112665 (*Z. h. campestris*) had haplotype KU45, specimen KU109963 had haplotype ZhcCR_A and specimen KU110033 was found to have a unique haplotype not previously reported by REA or KEA.

Haplotype L/PAL/C2

Haplotype L/PAL/C2 was reported to be shared among three subspecies of *Z. hudsonius* by REA. Nine specimens are listed as having haplotype L/PAL/C2 by REA: six *Z. h. luteus* (MSB86344, MSB91627, MSB91675, NK1584, DMNH8635, and DMNH8633), two *Z. h. campestris* (KU41451, KU112661), and one *Z. h. pallidus* (KU153706). Of these nine

specimens only three (NK1584, DMNH8635, DMNH8633) have consistent numbering between Appendix 2 of REA and data accessioned in GenBank. When the REA GenBank data is compared to that of KEA, NK1584, DMNH8635 and DMNH8633 were not found to match any haplotypes, but did match a haplotype from REA. This haplotype is referred to as KU45; based on a specimen code in GenBank (REA accession) for an individual of *Z. h. pallidus* with this haplotype. Eight other specimens from GenBank were also found to match this haplotype: six *Z. h. luteus* (MSB8, MSB7, MSB5, NK1584, DMNH8635, and DMNH8633) and two *Z. h. campestris* (KU27, KU1). Given this evidence we believe haplotype L/PAL/C2 of REA is identical to haplotype KU45 of the REA GenBank accession. Tissue samples from two specimens (KU153706 and KU112661) were obtained from the KUMNH. Specimen KU153706 was found to have haplotype KU45 as reported by REA. However specimen KU112661 (*Z. h. campestris*) had haplotype ZhcCR_D, which is not the same haplotype as that reported by REA (haplotype KU45).

Haplotype C9/INT-VII

Haplotype C9/INT-VII was reported by REA to be shared by *Z. h. campestris* and *Z. h. intermedius*. Two specimens were listed by REA as having C9/INT-VII, KU112663 (*Z. h. campestris*) and KU115730 (*Z. h. intermedius*). Only KU115730 had consistent numbering between Appendix 2 of REA and data accessioned in GenBank. When the REA GenBank data was compared to that of KEA, KU115730 was not found to match any haplotype. One other REA sequence, KU26 (*Z. h. campestris*), had the C9/INT-VII haplotype, leading us to believe that KU26 of the GenBank alignment is KU115730. Tissue samples from the two specimens (KU112663 and KU115730) were obtained from the KUMNH. Both specimens, KU112663 and KU115730 were found by KEA to have haplotype ZhcCR_D, not C9/INT-VII as reported by REA.

Haplotype C5/INT-XIII

REA report haplotype C5/INT-XIII as being shared between *Z. h. campestris* and *Z. h. intermedius*. Specimens KU87040, KU83557, and KU87042 were collected from Harding County, South Dakota and catalogued by the museum as *Z. h. campestris*. KU115895, KU115896, and KU115897 were collected from the same general location in Harding County, South Dakota but were identified as *Z. h. intermedius*. The respective collectors of both sets of samples collaborated in a publication (Anderson & Jone 1971) in which they named all *Z. hudsonius* from this location as *Z. h. campestris*. This haplotype is not shared between the two subspecies.

Table B.1 provides the results of the validation for the eight additional museum skins. In total, we investigated eight of 10 haplotypes reported by REA to be shared between or among selected subspecies of *Z. hudsonius*. Of the 15 specimens analysed, 13 were found to have haplotypes distinctly different from those reported by REA. We believe these findings have identified the presence of a systemic error in the CR data reported by REA. Moreover, the inability to directly link the sequences deposited in GenBank to the specimens listed in Appendix 2 of REA and the associated locality data renders the data useless. Possible reasons for the different sequences reported by REA are contamination, mislabelling of samples, or other procedural incongruity. Given that all the specimens re-evaluated in the current study were found by REA to have haplotypes that were shared among subspecies, the disagreement in DNA sequences reported for these individuals calls into question all of the results of REA based on the mitochondrial DNA genome and prevents analysis of the combined data.

Appendix C

Microsatellite DNA markers, the total number of alleles observed, and the range of amplified products in the survey of 348 *Zapus hudsonius*, and associated references

Microsatellite locus	Alleles observed	Size range	Reference
Z.7	30	156–179	Ramey *et al*. (2005)
Z.20	20	103–147	Ramey *et al*. (2005)
Z.26	16	138–174	Ramey *et al*. (2005)
Z.48	16	173–203	Ramey *et al*. (2005)
Z.52	11	154–176	Ramey *et al*. (2005)
*Ztri*2	12	91–135	Vignieri (2003)
*Ztri*17	14	149–195	Vignieri (2003)
*Ztri*19	8	174–206	Vignieri (2003)
*Ztri*24	13	151–199	Vignieri (2003)
*Zhu*C3	16	204–264	King *et al*. (2006)
*Zhu*C6	10	100–144	King *et al*. (2006)
*Zhu*C12	8	96–124	King *et al*. (2006)
*Zhu*C104	11	222–254	King *et al*. (2006)
*Zhu*C119	17	207–263	King *et al*. (2006)
*Zhu*C120	7	145–169	King *et al*. (2006)
*Zhu*C129	10	200–236	King *et al*. (2006)
*Zhu*C130	7	258–286	King *et al*. (2006)
*Zhu*D107	13	213–261	King *et al*. (2006)
*Zhu*D108	10	138–176	King *et al*. (2006)
*Zhu*D109	13	133–177	King *et al*. (2006)
*Zhu*D122	18	201–285	King *et al*. (2006)

Appendix D

Zapus hudsonius mitochondrial DNA control region haplotype (372 base pairs) counts by subspecies and collection

	Z. hudsonius preblei							*Z. h. intermedius*		*Z. h. campestris*		*Z. h. pallidus*		*Z. h. luteus*
Haplotype	SOWY	LCCO1	LCCO2	DCCO1	DCCO2	ECCO1	ECCO2	BRCSD	MCMN	CCWY	CCSD	BCSD	KBCNE	SCNM
N	28	12	16	32	26	19	26	26	21	29	31	16	31	19
ZhpCR_A	28	12	12											
ZhpCR_B				6	18	9	11							
ZhpCR_C			4	26	5									
ZhpCR_D					3	10	15							
ZhiCR_A								8	4					
ZhiCR_B								5						
ZhiCR_C								13						
ZhiCR_D									8					
ZhiCR_E									3					
ZhiCR_F									2					
ZhiCR_G									1					
ZhiCR_H									2					
ZhiCR_I									1					
ZhcCR_A										25	20			
ZhcCR_B										3				
ZhcCR_C										1				
ZhcCR_D											8			
ZhcCR_E											3			
ZhpaCR_A												14	10	
ZhpaCR_B												2		
ZhpaCR_C													10	
ZhpaCR_D													7	
ZhpaCR_E													4	
ZhlCR_A														17
ZhlCR_B														2

Appendix E

Zapus hudsonius mitochondrial DNA cytochrome *b* region haplotype (1006 base pairs) counts by subspecies and collection.

	Zapus hudsonius preblei							*Z. h. intermedius*		*Z. h. campestris*		*Z. h. pallidus*		*Z. h. luteus*
Haplotype	SOWY	LCCO1	LCCO2	DCCO1	DCCO2	ECCO1	ECCO2	BRCSD	MCMN	CCWY	CCSD	BCSD	KBCNE	SCNM
N	28	14	15	31	14	19	25	26	21	30	30	16	32	19
ZhpCB_A		1	5											
ZhpCB_B		1												
ZhpCB_C		1												
ZhpCB_D		1												
ZhpCB_E		1												
ZhpCB_F		9	5											
ZhpCB_G	9		4											
ZhpCB_H			1											
ZhpCB_I				7	11	14	24							
ZhpCB_J				23										
ZhpCB_K				1		1								
ZhpCB_L					2									
ZhpCB_M					1									
ZhpCB_N						1								
ZhpCB_O						1								
ZhpCB_P						2								
ZhpCB_Q							1							
ZhpCB_R	5													
ZhpCB_S	1													
ZhpCB_T	1													
ZhpCB_U	12													
ZhiCB_A									3					
ZhiCB_B									1					
ZhiCB_C								5	4					
ZhiCB_D									1					
ZhiCB_E									3					
ZhiCB_F									1					
ZhiCB_G									5					
ZhiCB_H									1					
ZhiCB_I									2					
ZhiCB_J								13						
ZhiCB_K								6						
ZhiCB_L								1						
ZhiCB_M								1						
ZhcCB_A										1				
ZhcCB_B										22	19			
ZhcCB_C										1				
ZhcCB_D										1				
ZhcCB_E										2				
ZhcCB_F										1				
ZhcCB_G										1				
ZhcCB_H											2			
ZhcCB_I										1				
ZhcCB_J											5			
ZhcCB_K											1			
ZhcCB_L											2			
ZhcCB_M											1			
ZhpaCB_A												4		
ZhpaCB_B												9		
ZhpaCB_C												2	11	
ZhpaCB_D												1		
ZhpaCB_E													10	
ZhpaCB_F													6	
ZhpaCB_G													4	
ZhpaCB_H													1	
ZhlCB_A														19