Animal Conservation (2005) **8**, 329–346 © 2005 The Zoological Society of London. Printed in the United Kingdom doi:10.1017/S1367943005002313

Genetic relatedness of the Preble's meadow jumping mouse (*Zapus hudsonius preblei*) to nearby subspecies of *Z. hudsonius* as inferred from variation in cranial morphology, mitochondrial DNA and microsatellite DNA: implications for taxonomy and conservation

Rob Roy Ramey II[1,†], Hsiu-Ping Liu[1,2], Clinton W. Epps[3], Lance M. Carpenter[1] and John D. Wehausen[4]

[1] Denver Museum of Nature and Science, Department of Zoology, 2001 Colorado Blvd., Denver, Colorado 80205-5798, USA
[2] University of Denver, Department of Biological Sciences, 2190 E. Iliff, Denver, CO 80208, USA
[3] University of California, Department of ESPM, 137 Mulford Hall, Berkeley, CA 94720-3114, USA
[4] University of California, White Mountain Research Station, 3000 East Line Street, Bishop, CA 93514, USA

(First received 5 August 2004; Resubmitted 9 March 2005; accepted 11 May 2005)

Abstract

The Preble's meadow jumping mouse (*Zapus hudsonius preblei*) is listed as a threatened subspecies under the United States Endangered Species Act (US-ESA). The quantitative description of this subspecies was based on cranial measurements of only three adult specimens. It is one of twelve subspecies of *Z. hudsonius* and is a peripheral population at the western edge of its range. We tested the uniqueness of *Z. h. preblei* relative to other nearby subspecies of *Z. hudsonius* using a hypothesis testing approach and analyses of cranial morphometric, mtDNA sequence and nuclear microsatellite data obtained from museum specimens and archived tissues. Morphometric analysis of variance did not support the original description of *Z. h. preblei* as a subspecies. Principal component analysis of these data showed *Z. h. preblei* within the range of variation found in *Z. h. campestris* and *Z. h. intermedius*. Discriminant analysis correctly classified only 42% of *Z. h. preblei* skulls at jackknifed posterior probabilities >0.95 relative to *Z. h. campestris*. All mtDNA haplotypes found in *Z. h. preblei* were also found in *Z. h. campestris*. Simulation based estimates of current and historical gene flow (MDIV) revealed low, but non-zero, mtDNA gene flow among *Z. h. preblei* and several nearby subspecies. Analyses of five nuclear microsatellite loci using population pairwise F_{ST}, BAPS and STRUCTURE were consistent with morphometric and mtDNA results. These revealed low levels of genetic structure and evidence of recent gene flow and bottlenecks in *Z. h. preblei*. Due to a lack of clearly recognisable genetic, morphological, or adaptive differences, we synonymise *Z. h. preblei* and *Z. h. intermedius* with *Z. h. campestris*. We suggest that candidates for listing under the US-ESA, or similar biodiversity laws, be evaluated for genetic and/or morphological uniqueness to prevent the misallocation of resources to non-distinct taxa like *Z. h. preblei*.

INTRODUCTION

The United States Endangered Species Act (US-ESA) is intended to protect organisms that are threatened with extinction and promote their recovery. Organisms 'listed' for protection can include species, subspecies and distinct vertebrate population segments. Since the enactment of the US-ESA in 1973, 1851 organisms have been listed as threatened or endangered. Thirty-five organisms have since been removed from the list. Seven 'delistings' resulted from correction of taxonomic errors and six from recognition of other types of errors, while 14 organisms recovered and eight went extinct (http://ecos.fws.gov/tess_public/TESSWebpage). One of the criticisms of the US-ESA is that listings are sometimes based on antiquated taxonomy or weak inference (National Research Council, 1995; Cronin, 1997; Gordon, Lacy & Streeter, 1997). It is in the best interest of biodiversity conservation to evaluate the systematics and taxonomy of candidates for listing and delisting. If defensible data are lacking and a protected organism is not distinguishable with a high degree of certainty from neighbouring, non-threatened relatives, considerable financial and logistical conservation effort may be misallocated at the expense of other endangered organisms. This applies to biodiversity laws globally.

The Preble's meadow jumping mouse (*Zapus hudsonius preblei*) was listed as a threatened subspecies under the US-ESA in 1998 (US Fish & Wildlife Service, 1998). It is one of 12 subspecies of the meadow jumping mouse (*Z. hudsonius*), a species whose range covers approximately half of North America (Fig. 1). *Zapus hudsonius* are hibernators and generalists in their food and

[†]All correspondence to: Rob Roy Ramey II. Tel: +1 303 370 6443; Tel/Fax: +1 303 258 9535; E-mail: ramey@spot.colorado.edu

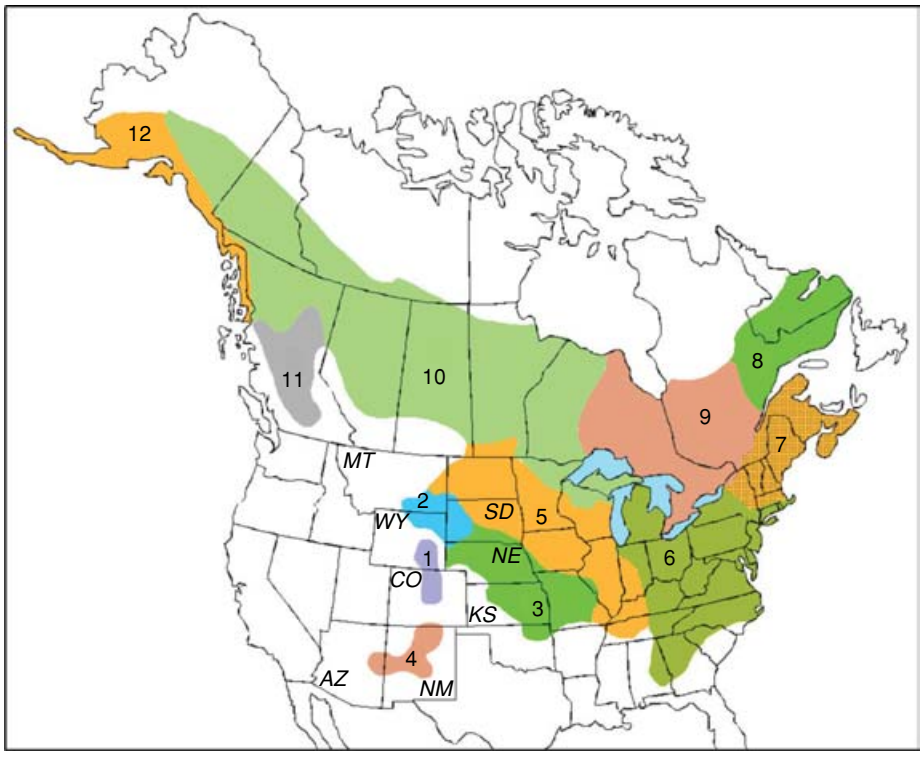

Fig. 1. Map of North America showing distribution and subspecies of *Zapus hudsonius* (Krutzsch, 1954; Hafner *et al*., 1981). (1) *Z. h. preblei*, (2) *Z. h. campestris*, (3) *Z. h. pallidus*, (4) *Z. h. luteus*, (5) *Z. h. intermedius*, (6) *Z. h. americanus*, (7) *Z. h. acadicus*, (8) *Z. h. ladas*, (9) *Z. h. canadensis*, (10) *Z. h. hudsonius*, (11) *Z. h. tenellus* and (12) *Z. h. alascensis*.

habitat preferences. They typically occupy moist habitats (e.g. meadows, marshes, bogs, streams and irrigation ditches) and adjacent drier areas including coniferous and hardwood forests, sand dunes, strip-mined land and tundra (Jones 1981). They are vagile compared to other small rodents (Quimby, 1951; Whitaker, 1972). Krutzsch (1954) described *Z. h. preblei* as a separate subspecies from the prairie jumping mouse (*Z. h. campestris*) based on skull measurements of three adult specimens and a qualitative description of four adult and seven juvenile skins. In contrast, Jones (1981) concluded that there were no valid subspecies of *Z. hudsonius* based on univariate morphometric analyses, a lack of distinguishing pelage differences, or plausible isolating mechanisms. Despite the weakness of Krutzsch's taxonomic inference by modern standards and the subsequent opposite conclusions reached by Jones (1981), the presumed uniqueness of *Z. h. preblei* based on morphological characters and geographical isolation was an important part of the decision to list it under the US-ESA. Less than 160 km of short grass prairie and agricultural land are presumed to separate *Z. h. preblei* from *Z. h. campestris* to the north, from *Z. h. pallidus* to the east and from *Z. h. luteus* to the south (Fig. 1).

Here, we test the uniqueness of *Z. h. preblei* relative to other nearby subspecies of *Z. hudsonius* using tests for multiple, genetically-based traits (Wehausen & Ramey, 2000; Pearse & Crandall, 2004). We treated taxonomic categories as testable hypotheses and used critical tests laid out in advance of data collection to provide an objective evaluation of the genetic distinctiveness of *Z. h. preblei* from nearby subspecies of *Z. hudsonius*. First, we retested the original quantitative basis of Krutsch's (1954) conclusions to split *Z. h. campestris* into three subspecies (*Z. h. preblei*, *Z. h. campestris* and *Z. h. intermedius*) using univariate and multivariate statistical analyses of skull measurements. Second, we used the conceptual approaches of Ball & Avise (1992), Avise & Johns (1999) and Hendry *et al*. (2000) as the basis of additional tests of *Z. h. preblei* as a subspecies. These authors and others (Crandall *et al*., 2000; Zink, 2004) have sought consistency by suggesting that taxa or distinct populations be defined by congruence of multiple genetically-based traits. This is also important because phenotypic variation can reflect both genetic and environmental influences (Keita *et al*., 2004). Third, we tested genetic and ecological exchangeability (Crandall *et al*., 2000) of *Z. h. preblei* relative to other subspecies to determine if it should be considered a distinct population and, therefore, a conservation priority.

Although it has long been recognised that many named subspecies are questionable (Wilson & Brown, 1953), it has also been recognised that subspecies classification can have some conservation utility if it has an evolutionary basis (Avise & Ball, 1990). Ball & Avise (1992) proposed that subspecies represent a major division in the gene pool diversity of a species based on concordant distributions of multiple genetically-based traits and have a plausible evolutionary mechanism for differentiation. These criteria are similar to those suggested for Evolutionary Significant Units by some authors (Fraser & Bernatchez, 2001). Hendry *et al*. (2000) proposed that conservation priority be afforded to populations that show greater genetic diversity among, relative to within, populations. We satisfied these

requirements using tests of uniqueness for multiple genetically-based traits including cranial morphometric data, mtDNA sequences and microsatellite markers. We required that at least two of the three data sets be considered corroborating evidence.

Crandall *et al*. (2000) proposed a hypothesis testing approach for recognising distinct populations at several levels, using the criteria of genetic and ecological exchangeability on recent and historic time scales. They proposed that ecological differences among populations could reflect adaptive differences that would not be detected by molecular markers alone. Therefore, we examined the literature for evidence of adaptive differences (e.g. life history, morphology) between subspecies and tested for potential shape differences in cranial morphology using principal components analysis (PCA) and linear discriminant analysis (LDA). We estimated the extent of current gene flow for mtDNA (using MDIV) and divergence at presumably neutral microsatellite loci (using pairwise genetic distances, F_{ST} and assignment tests).

We attempted to use threshold levels for various tests (AMOVA, LDA, etc) that have some conventional history below the level of species (e.g. Worley *et al*.'s 2004 use of $q > 0.90$ as a standard in assignment tests; Wehausen & Ramey's 2000 use of >0.90 correct assignment using posterior probabilities of $P > 0.95$ in LDA on morphometric data). Any such threshold level (such as the $P < 0.05$ test for significance commonly employed in frequentist statistics) can be seen as arbitrary; however, we hope to establish reasonable threshold levels for these sorts of tests where they have often been absent. Systematic decisions rely on distinguishability among groups at hierarchical levels (Avise & Johns, 1999). In the case of endangered taxa or populations, a higher certainty of correct assignment and congruence among data sets suggests a higher degree of genetic uniqueness and conservation priority. Appropriate thresholds can be debated and revised, but we feel that the first step in establishing standards and objectively applying them is to state them explicitly.

MATERIALS AND METHODS

Cranial morphometrics

We retested the quantitative basis of Krutsch's (1954) conclusions regarding cranial differences between *Z. h. preblei* and *Z. h. campestris* using the same nine skull measurements: occipitonasal length, condylobasal length, palatal length, zygomatic length, zygomatic breadth, mastoidal breadth, braincase breadth, interorbital breadth and upper tooth row length. Skulls were from collections at the Denver Museum of Nature & Science (DMNS) and the University of Kansas Museum of Natural History (KU) (Appendix 1). Identity of samples was from museum tags, which relied upon geographical area from which a sample was collected and the current subspecies classification (Krutzsch, 1954; Hafner, Peterson & Yates, 1981). For each variable, four repeated measurements were taken using digital calipers and recorded to the nearest hundredth of a millimetre. Only adult skulls were measured, as determined by all molars being completely erupted and having slight wear on M^3 (Krutzsch, 1954). Fewer measurements were taken for some specimens due to incomplete material. Calipers were moved away from the skull and reset for each measurement. One person (L.M.C.) measured all skulls (Palneirim, 1998). We used the means of the repeated measurements for 40 *Z. h. preblei* and 40 *Z. h. campestris* in ANOVA, PCA and LDA (Conner & Shenk, 2003). Those two subspecies were then combined for comparisons with 37 *Z. h. intermedius*.

The critical test of the original subspecies description was two-fold. First, the hypothesis of *Z. h. preblei* being a unique, smaller subspecies relative to *Z. h. campestris* would be rejected if the skulls of *Z. h. preblei* were not significantly smaller for the majority of skull measurements. Second, we used LDA to test uniqueness with the distinguishability criterion that $\geq 90\%$ of the specimens be correctly classified to subspecies at jackknifed posterior probabilities ≥ 0.95 (Wehausen & Ramey, 2000). This unambiguous criterion requires that specimens be correctly classified with a high degree of certainty using a multivariate analysis of shape. Outliers were removed using Grubb's and Dixon's tests (Sokal & Rohlf, 1981) and stepwise procedures were used to limit the model to discriminating variables for 33 *Z. h. preblei* and 39 *Z. h. campestris* that had complete measurements. We also used the combined sample of *Z. h. preblei* and *Z. h. campestris* for comparison with *Z. h. intermedius* ($n = 37$). Males and females were pooled because of an apparent lack of sexual dimorphism (Jones, 1981; Conner & Shenk, 2003). Incomplete specimens could not be used in LDA if any variable used in the model was missing for that specimen.

Krutzsch's qualitative descriptions of skull shape and pelage, that presumably distinguished *Z. h. preblei* from *Z. h. campestris*, included: incisive foramina not truncate posteriorly; auditory bullae smaller and less well inflated; frontal region usually more inflated; upper parts generally dull, averaging lighter; sides duller; less black tipped hair. These subjective criteria and those describing *Z. h. campestris* relative to *Z. h. intermedius* (coloration more tawny and ochraceous, less yellow; auditory bullae averaging larger, more inflated; incisive foramina not truncate posteriorly), were not readily quantifiable and were not used in subsequent analyses.

We used PCA as an exploratory tool to look for geographical patterns in cranial size and shape variation across the study area and to identify variables that contributed strongly to any components that showed geographical separation (Reyment, 1990). We ran PCA on the nine cranial characteristics. PCA was performed on covariance matrices derived from pairwise analyses of natural-log-transformed variables (Reyment, Blackith & Campbell, 1984).

MtDNA sequencing

We analysed a segment of highly variable mitochondrial DNA control region sequence for 205 museum skins or liver tissues of *Z. hudsonius* (58 *Z. hudsonius preblei*, 33 *Z. h. campestris*, 32 *Z. h. luteus*, 35 *Z. h. pallidus* and 47 *Z. h. intermedius*) (Appendix 2). For outgroup comparison

we used 17 specimens of western jumping mouse (*Z. princeps*) (Appendix 3). Specimens were obtained for genetic analysis from museum collections at DMNS, KU, the Nebraska State Museum (NSU) and the University of New Mexico Museum of Southwestern Biology (MSB). We sampled across the range of each putative subspecies in order to assess the amount of genetic variation within a subspecies. Thus, we sampled more locations but fewer individuals per location.

Genomic DNA was extracted from frozen liver tissue, museum skin samples (5–10 mg) and ear punch specimens using Qiagen DNeasy Tissue kit (Qiagen Inc.). Approximately 460 base-pairs (bp) of the mitochondrial control region were amplified via polymerase chain reaction (PCR) using the primers L15320 (5'ATAAACATTACTCTGGTCTTGTAAACC3′) and ZAP5P1r (5′ATGGCCCTGAAGTAAGAACCAG3′). Amplifications were conducted in a 25 μl total volume, containing 5 μl of Invitrogen optimiser buffer D (17.5 mM $MgCl_2$, pH 8.5) (Invitrogen, Inc.), 2.5 μl of dNTPs (2.5 mM each), 1.25 μl of each primer (10 μM), one unit *Taq* polymerase, one μl of template (*ca*. 50–100 ng double-stranded DNA) and 13.8 μl of sterile water. Thermal cycling was performed with an initial denaturation for 2 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 58 °C, 2 min at 72 °C, with a final extension of 10 min at 72 °C. Some museum specimens were amplified using nested PCR. We designed the nested primers, L15398 (5′ATCAGCACCCAAAGCTGATATTC3′) and H16498 (5′CCTGAAGTAAGAACCAGATG3′), which amplified roughly 385bp within the first amplicon. Nested PCR was performed by using 1.0 μl of the PCR product from the first reaction mixture as a template and reamplifying it with the nested pairs of primers. The remaining procedure was identical to the first PCR. Multiple negative controls were run with every PCR batch. The amplified PCR product was cleaned using the Exo/SAP method. Double-stranded DNA templates were incubated at 37 °C for 30 min and then at 85 °C for another 15 min with five units of Exonuclease I (ExoI, Amersham) and 0.5 unit Shrimp Alkaline Phosphatase (SAP, Amersham). For cycle sequencing reactions we used 1–5 μl (20ng) of the cleaned PCR product as a template in a 10 μl total volume with the CEQ DTCS Quick Start Kit (Beckman Coulter, Inc.). The following cycling conditions were used: 96 °C for 2 min, then 30 cycles of 96 °C for 20s, 50 °C for 20s and 60 °C for 2 min. The cycle-sequenced products were purified using an ethanol precipitation method following the Beckman Coulter protocol and separated by electrophoresis using a Beckman Coulter CEQ8000 sequencer.

Consensus sequences were aligned using Sequencher 3.1.1 (Gene Codes Corp., Ann Arbor, MI) and verified manually. Phylogenetic analyses based on distance, parsimony and maximum-likelihood methods were conducted using PAUP* 4.0b10 (Swofford, 2002). Modeltest 3.06 (Posada & Crandall, 1998) was used to evaluate 56 models of evolution in order to obtain an appropriate substitution model and parameter values for distance and maximum-likelihood analyses. Appropriate genetic distance (based on Modeltest results) was used to generate neighbour-joining (NJ) trees based on the clustering method of Saitou & Nei (1987). Node support was assessed by completion of 10 000 bootstrap replications (Felsenstein, 1985) in PAUP*, using the fast-search option. Maximum-parsimony (MP) analyses were conducted with equal weighting, using the heuristic search option with tree bisection reconnection branch-swapping, 100 replications of random stepwise additions, gaps treated as missing, and MAXTREES set to 10 000. Bootstrapping with 10 000 replications (as implemented in PAUP*) was used to evaluate node support. The most likely model selected by Modeltest was used for maximum likelihood (ML) analyses. A neighbour-joining tree with appropriate genetic distance was used as the initial topology for branch-swapping. Node support was evaluated by 100 bootstrap pseudoreplicates. Split decomposition (SD) was calculated using SplitsTree version 2.4 (Huson, 1998) for all mtDNA data and for *Z. hudsonius* mtDNA data alone. Branch support was evaluated using 50 bootstrap replications.

Four *Z. hudsonius* specimens from Wyoming, one from Kansas, one from Montana and one from South Dakota had mtDNA haplotypes nearly identical to the highly divergent haplotypes found in *Z. princeps*. These seven specimens were presumed to be misidentified and were excluded (Appendix 3). *Zapus hudsonius* and *Z. princeps* are difficult to distinguish from pelage alone, although the latter tend to be larger. In order to provide a reasonable tree size, one sequence from each haplotype of *Z. hudsonius* and one representative sequence from each *Z. princeps* subspecies were used in all phylogenetic analyses.

ARLEQUIN 2.0 was used to perform an analysis of molecular variance (AMOVA) to partition the amount of genetic variation in a hierarchical fashion within and between the subspecies (Excoffier, Smouse & Quattro, 1992). MEGA 2 (Kumar *et al*., 2002) was also used to estimate mtDNA nucleotide diversity. Tajima's *D* was used as a test of selective neutrality for mtDNA using ARLEQUIN 2.0.

Our critical test of uniqueness for *Z. h. preblei* using mitochondrial DNA sequence data was that there be greater molecular variance among than within subspecies (in pairwise comparisons involving *Z. h. preblei*) or that samples show nearly complete reciprocal monophyly with respect to other subspecies.

MtDNA MDIV

Fixation indices such as F_{ST} (Wright, 1921) are calculated under assumptions of equilibrium; any shared genetic variation is therefore assumed to be the result of current gene flow. Thus F_{ST} cannot distinguish between recently-isolated populations with no gene flow and populations isolated for a longer period of time but with continuing low levels of gene flow. As an alternative, we used the maximum-likelihood based program MDIV (Nielsen & Wakeley, 2001) to evaluate whether shared mtDNA variation between *Z. h. preblei*, *Z. h. campestris, Z. h. intermedius, Z. h. pallidus* and *Z. h. luteus* reflected very

Table 1. Dinucleotide microsatellite primers used in this study

Locus	GenBank accession no.	Primer sequence (5′ to 3′)	Annealing temp (°C)	Repeat of cloned allele	No. of alleles	Allele size range
Z.20	DQ063596	F:TCTTCCTCCCCCAGACCTAC R:TCCCAAGGCCTAAACAGTGA	60	$(CA)_9$	20	109–149
Z.48	DQ063597	F:GCTCATCTGCAATGGAGGA R:TTGTCTTTAGAAACAAGATTTACT	60	$(CA)_{23}$	18	182–210
Z.52	DQ063598	F:CCTCCCAGCTCTGTCTTTGA R:TGGACAAGGCTACTGCTTCC	60	$(GT)_{21}$	13	155–181
Z.7	DQ063599	F:CTTAGGCCTTGCAGTCAAGC R:TTAGCACTTCCAGCACATGG	60	$(GT)_7$	20	154–190
Z.26	DQ063600	F:CATTTTACACCAGCAAACAGG R:TATTGGCTGCACATTCTTGC	60	$(CA)_{16}$	19	141–171
Z.47	DQ063601	F:TGAAAAGAGCTAAATACTTGGGTAGA R:TGTCATTGCTCACTGTTTCCA	60	$(CA)_{24}$	15	121–149

recent (including current) gene flow or complete, but recent, isolation.

MDIV uses Markov-chain Monte Carlo simulations to estimate for two populations the likelihood of the parameters *theta* ($4N_e\mu$) and *M* ($2N_em$), where N_e is the effective population size, *m* is the migration rate and μ is the mutation rate. MDIV assumes that N_e and *m* are the same for both populations. We used MDIV to estimate migration (*m*) between *Z. h. preblei* and *Z. h. campestris* and to compare this estimate of gene flow to estimates of gene flow between other pairs of populations. We ran 5 000 000 chains for each simulation with burn-in of 500 000 chains, set T_{MAX} and M_{MAX} to 10 and used the HKY model of sequence evolution (a software constraint). Parallel simulations gave similar results, suggesting that this number of chains was adequate. MDIV tests a wide range of values for each parameter and calculates the likelihood of each tested value. We calculated confidence intervals around the parameter estimates using Akaike's Information Criterion (AIC: Burnham & Anderson, 1998) to determine the range of parameter values that were not significantly less likely than the best estimated value (Nielsen & Wakeley, 2001). Because the number of parameters was fixed, we calculated AIC as:

$$\mathrm{AIC} = -2^* \log\ (\text{likelihood})$$

We accepted parameter values within 2 AIC units on either side of the most likely estimated parameter value. However, due to the assumptions made by MDIV about N_e and *m*, these confidence intervals may be understated. We calculated N_e from the estimate of theta using $\mu = 2.5{*}10^{-5}$ over 346 bp (estimated from divergence in vole mtDNA control region sequences by Matson & Baker (2001)). We converted *M* to *m* using the most likely estimate of N_e.

Microsatellites

Six dinucleotide-repeat microsatellite loci (Table 1) were isolated and sequenced using methods described previously (Oyler-McCance *et al*., 2005). The amplification, electrophoresis and scoring methods used were as previously described (Wehausen, Ramey & Epps, 2004).

We estimated allelic richness (*A*) using FSTAT (Goudet, 1995) to correct for variation in sample size, as recommended by Leberg (2002). Allelic richness can be a sensitive comparative indicator of population bottlenecks or founder effects (Leberg, 2002). We also used FSTAT to calculate F_{IS} values within populations and to test for linkage disequilibrium within populations within loci and within loci across populations. Population pairwise F_{ST} values (Weir & Cockerham, 1984) were calculated by GENEPOP (Raymond & Rousset, 1995). Our critical test of uniqueness for subspecies and historic genetic exchangeability (Crandall *et al*., 2000) was two-fold: that there be greater variation between *Z. h. preblei* and other subspecies than within each subspecies in pairwise comparisons (using F_{ST} and AMOVA) and that multiple private alleles be at higher frequency than shared alleles at the majority of loci. We do not claim that these criteria alone can be used to define subspecies, or that they are universally applicable, merely that they provide an unambiguous test of deeply historic genetic divergence among populations.

We used BAPS (Corander, Waldmann & Sillanpaa, 2003) to examine genetic clustering of putative subspecies and 'populations'. We also used it to estimate Nei's genetic distance (*D*) between putative subspecies and between populations. We used the 'population' analyses to compare variation between biogeographically-relevant groupings within putative subspecies with variation between putative subspecies. We divided *Z. h. preblei* into 'North' and 'South' populations, based on a suspected biogeographical split imposed by the Denver metropolitan area. We divided *Z. h. intermedius* into 'West' (North Dakota, South Dakota, western Iowa) and 'East' populations (central and eastern Iowa, Illinois and Indiana). This divided the range approximately in half. We treated *Z. h. campestris*, *Z. h. pallidus* and *Z. h. luteus* as discrete populations in this analysis. We estimated *D* using the multiple-chain McMC approach. We set burn-in time to 15 000, chain length to 50 000, ran five chains, set thinning to 5 and checked to ensure that these values were sufficient to

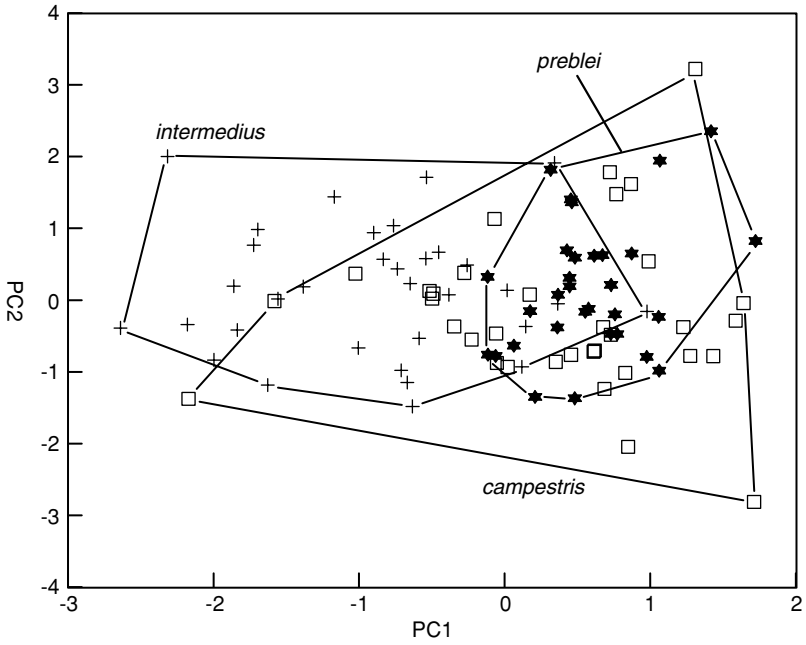

Fig. 2. Plot of Principal Component scores for PC1 and PC2. Subspecies are indicated by polygons.

achieve convergence. We used an initialisation of $k = 7$ clusters and, in the results state, set the minimum partition to 0.05.

We used STRUCTURE (Pritchard, Stephens & Donnelly, 2000) to attempt to determine how many clusters (k) were diagnosable in the combined data set of all 195 specimens. For each cluster number examined, STRUCTURE generates a likelihood value; the maximum value indicates the most likely cluster number. We tested $k = 1$ through $k = 15$, using a burn-in of 15 000 followed by 100 000 replications. Using the cutoff value of $q = 0.90$ (where q is the likelihood of assignment of an individual to a given cluster), as suggested by Worley *et al*. (2004), we examined how many specimens of each population (with *Z. h. preblei* samples divided into southern and northern populations and *Z. h. intermedius* divided into eastern and western populations) were assigned with confidence greater than or equal to this cut-off value of q. Our critical test of recent genetic exchangeability using STRUCTURE was that clusters correspond to subspecies or populations – with a high level of correct assignment of individuals (>90%) using $q = 0.90$. This criterion rules out weakly differentiated populations as conservation priorities.

RESULTS

Testing the original quantitative basis of taxonomic categories

Krutzsch (1954) stated that *Z. h. preblei* was smaller than *Z. h. campestris* in 'most skull dimensions measured.' However, our results revealed that *Z. h. preblei* was significantly ($P < 0.05$) smaller for only one measurement (interorbital breadth), but larger for two measurements (zygomatic and mastoid breadth) and insignificantly different for the six others. The significant differences between subspecies were very small and of questionable biological significance relative to measurement resolution. The classification of *Z. h. preblei* as a separate subspecies therefore failed the test of uniqueness using the original criteria. When a combined sample of *Z. h. campestris* and *Z. h. preblei* was compared to *Z. h. intermedius*, they were significantly larger in all skull measurements. This is consistent with Krutzsch's description of *Z. h. intermedius* being slightly smaller, although Krutzsch noted substantial intergradation with *Z. h. campestris* and *Z. h. pallidus*. Measurement data used in morphometric analyses were deposited with the Archivist at DMNS and are available online at www.dmns.org.

PC1 explained 67.1% of the variation with positive loadings on all variables, suggesting that this is a general size component. PC2 accounted for 11% of the variance, mostly in tooth row length. PC3 accounted for 10.3% of the variance, mostly in interorbital breadth. When PC1 is plotted against PC2 on a pooled sample of *Z. h. preblei*, *Z. h. campestris* and *Z. h. intermedius*, *Z. h. preblei* specimens fall entirely within *Z. h. campestris* along the PC1 axis. *Zapus h. intermedius* however, is somewhat separable as smaller (Fig. 2). There is no subspecies separation on the PC2 axis or when PC3 and PC4 were plotted. While PCA on cranial measurements has limitations for inferring shape differences that are independent of size, there appears to be almost no difference between *Z. h. preblei* and *Z. h. campestris*. About half of the *Z. h. intermedius* specimens, however, appear to be smaller than *Z. h. preblei* and *Z. h. campestris*.

Four variables were determined to have the greatest discriminating power between *Z. h. campestris* and *Z. h. preblei*, using forward and backward stepwise procedures in LDA. These were zygomatic breadth, mastoidal breadth, breadth of skull and condylobasal length. Only 42% of the specimens could be classified correctly at

posterior probabilities ≥ 0.95, further indicating a lack of morphometric distinguishability of these two subspecies. We therefore rejected the hypothesis that *Z. h. preblei* is unique in cranial shape from *Z. h. campestris*.

Only mastoidal breadth and interorbital breadth contributed significantly to the discriminant function for the combined sample of *Z. h. preblei* and *Z. h. campestris* ($n = 73$) against *Z. h. intermedius* ($n = 35$). That function had poor discrimination ability, with only 31.5% of specimens being correctly classified at a jackknifed posterior probability of ≥ 0.95. We therefore rejected the hypothesis of uniqueness for *Z. h. campestris* and *Z. h. preblei* combined from *Z. h. intermedius*. In comparison, Conner & Shenk (2002) had found a high degree of classification certainty between species of jumping mice (*Z. princeps* and *Z. hudsonius*) in Colorado and Wyoming with >96% of specimens correctly classified at a posterior probability > 0.95.

Testing putative subspecies: MtDNA analyses

DNA sequences were deposited in GenBank with accession numbers AY598142 – AY598316 and AY971529 – AY971575. The final aligned data matrix for mtDNA analyses, including indels, was 346 bp, of which 68 (19.7%) sites were variable and 47 (13.6%) were parsimony informative. Values of Tajima's *D* were not significant ($P > 0.05$) for subspecies considered individually or pooled together. Therefore, the null hypothesis of selective neutrality for mtDNA could not be rejected. Nucleotide diversity ranged from 0.0027 in *Z. h. preblei* to 0.0215 in *Z. h. campestris* (Table 2). Forty-three haplotypes were observed for *Z. hudsonius*. Modeltest (version 3.06, Posada & Crandall, 1998) selected the TVM model (Transversional model, a variation of the General Time Reversible model (GTR)), with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution (e.g. TVM + I + G: Tavare, 1986; Posada & Crandall, 1998) as the best fit for the dataset using AIC. The optimised parameters were base frequencies of A = 0.2919, C = 0.2629, G = 0.0957, T = 0.3495; Rmat = {3.2955 24.2634 7.5746 0.8175 24.2634}; shape of gamma distribution = 0.6499; and proportion of invariant sites = 0.6174. GTR distance was used to generate NJ trees and the TVM + I + G model was used for ML analyses. Distance analysis (neighbour-joining tree), MP, ML and SD resolved haplotypes into two strongly supported *Z. hudsonius* lineages. These included a *Z. h. preblei/Z. h. campestris/Z. h. intermedius* lineage and a *Z. h. luteus/Z. h. pallidus* lineage (Fig. 3). The MP, ML and SD (not figured) topologies were congruent with the NJ tree (Fig. 3) and differed in the positioning of terminal taxa. SD analysis of *Z. hudsonius* mtDNA data supported the two lineages (100% bootstrap support) and unresolved polytomies for terminal branches with low bootstrap support (< 66%).

The number of variable nucleotides and haplotypes and nucleotide diversity for each subspecies are presented in Table 2. The four haplotypes that occurred in *Z. h. preblei* also occurred within the range of *Z. h. campestris*.

Table 2. MtDNA control region sequence diversity found in subspecies of *Zapus hudsonius*

Taxa	*N*	Variable sites	Haplotypes	Nucleotide diversity
Z. h. preblei	54	3	4	0.0027
Z. h. campestris	31	29	15	0.0215
Z. h. intermedius	47	31	16	0.0068
Z. h. luteus	32	6	8	0.0042
Z. h. pallidus	34	30	12	0.0138

These shared haplotypes span a range of up to 700 km, from central Colorado to western South Dakota and southeastern Montana. Four sequences (two haplotypes) of *Z. h. campestris* were grouped in the *Z. h. luteus*/*Z. h. pallidus* lineage. Nearly all of the *Z. h. intermedius* haplotypes (except one) were found in the *Z. h. preblei/Z. h. campestris/Z. h. intermedius* lineage (Figs 3 & 4). Four of the *Z. h. intermedius* haplotypes were identical to those found in *Z. h. campestris* (Fig. 3). *Zapus h. preblei* was not reciprocally monophyletic with respect to any other subspecies. Two sequences of *Z. h. pallidus* from Clay Co., South Dakota were more similar to sequences of *Z. h. campestris* and *Z. h. preblei* than to other sequences of *Z. h. pallidus*.

Analysis of molecular variance between *Z. h. preblei* and *Z. h. campestris* revealed that most of the genetic variation was within (63%) rather than between (37%) these putative subspecies. In the case of *Z. h. luteus* and *Z. h. pallidus* (separated by ~500 km), each has several unique haplotypes (6 and 9, respectively) but, as with *Z. h. preblei* and *Z. h. campestris*, most of the molecular variance was within (72%) rather than between (28%) these putative subspecies. In combination with the absence of any genetic structure that even approached reciprocal monophyly, these results led us again to reject the hypothesis of uniqueness of *Z. h. preblei* relative to *Z. h. campestris*.

When *Z. h. intermedius, Z. h. campestris* and *Z. h. preblei* were considered separately from *Z. h. pallidus* and *Z. h. luteus*, greater variation was found within (69.3%) than between (30.7%) the subspecies. When only *Z. h. intermedius* and *Z. h. campestris* were compared, considerably greater variation was found within (96.2%) than between those two subspecies (3.8%). When *Z. h. campestris* and *Z. h. preblei* were combined as a single subspecies and compared with *Z. h. intermedius*, only 18.5% of the variation was found between subspecies (81.5% within subspecies). Based on these analyses, we reject the hypothesis of uniqueness for *Z. h. intermedius* relative to *Z. h. campestris*.

Testing putative subspecies: microsatellites

Six microsatellite loci genotypes were recorded for 195 *Z. hudsonius* specimens for which mtDNA was sequenced (Appendix 2). One locus, .47, was not considered a reliable neutral genetic marker because it had very high

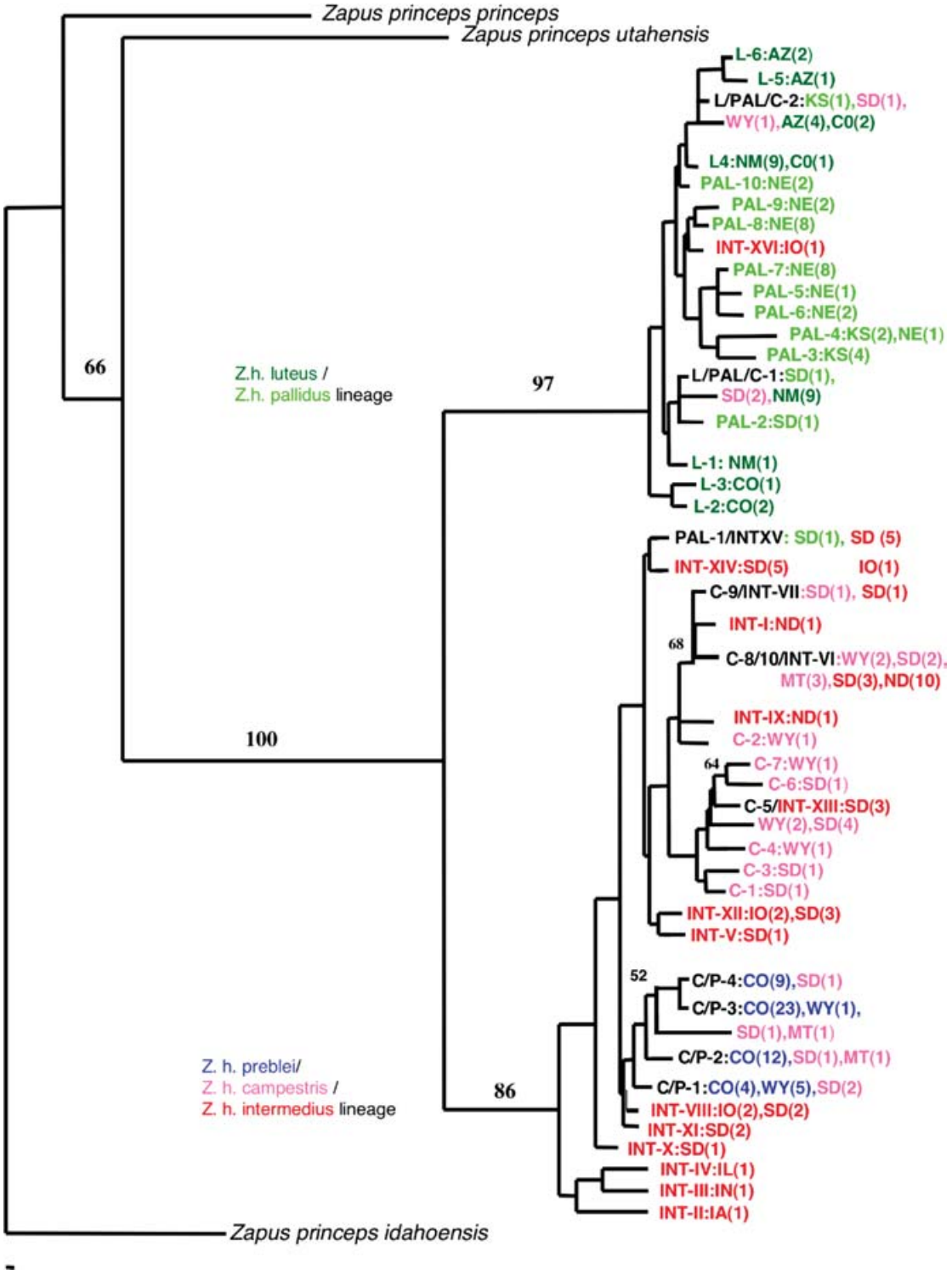

Fig. 3. Neighbour-joining phylogram inferred from partial mitochondrial DNA control region, depicting phylogenetic relationships between haplotypes of *Zapus hudsonius*. Bootstrap percentages are given when ≥ 50%. State and number of individuals with identical haplotypes are listed. Colours indicate subspecies (Krutzsch, 1954; Hafner *et al*., 1981) as follows: *Z. h. preblei* (blue), *Z. h. campestris* (pink), *Z. h. intermedius* (red), *Z. h. luteus* (dark green) and *Z. h. pallidus* (light green).

values of F_{IS} (0.69–0.94) and consistently violated Hardy–Weinberg equilibrium, suggesting the presence of null alleles or selection at closely linked loci. It was dropped from subsequent analyses. When the data set was divided into five putative subspecies or into seven populations, no significant linkage disequilibrium ($P < 0.05$, after correcting for multiple comparisons) was found in any population at any locus, or by locus across populations. F_{IS} for all populations was positive, with a pattern of heterozygote deficiency across most loci (Table 3). A probable explanation for the observed heterozygote deficiency is a Wahlund effect due to sampling of only one,

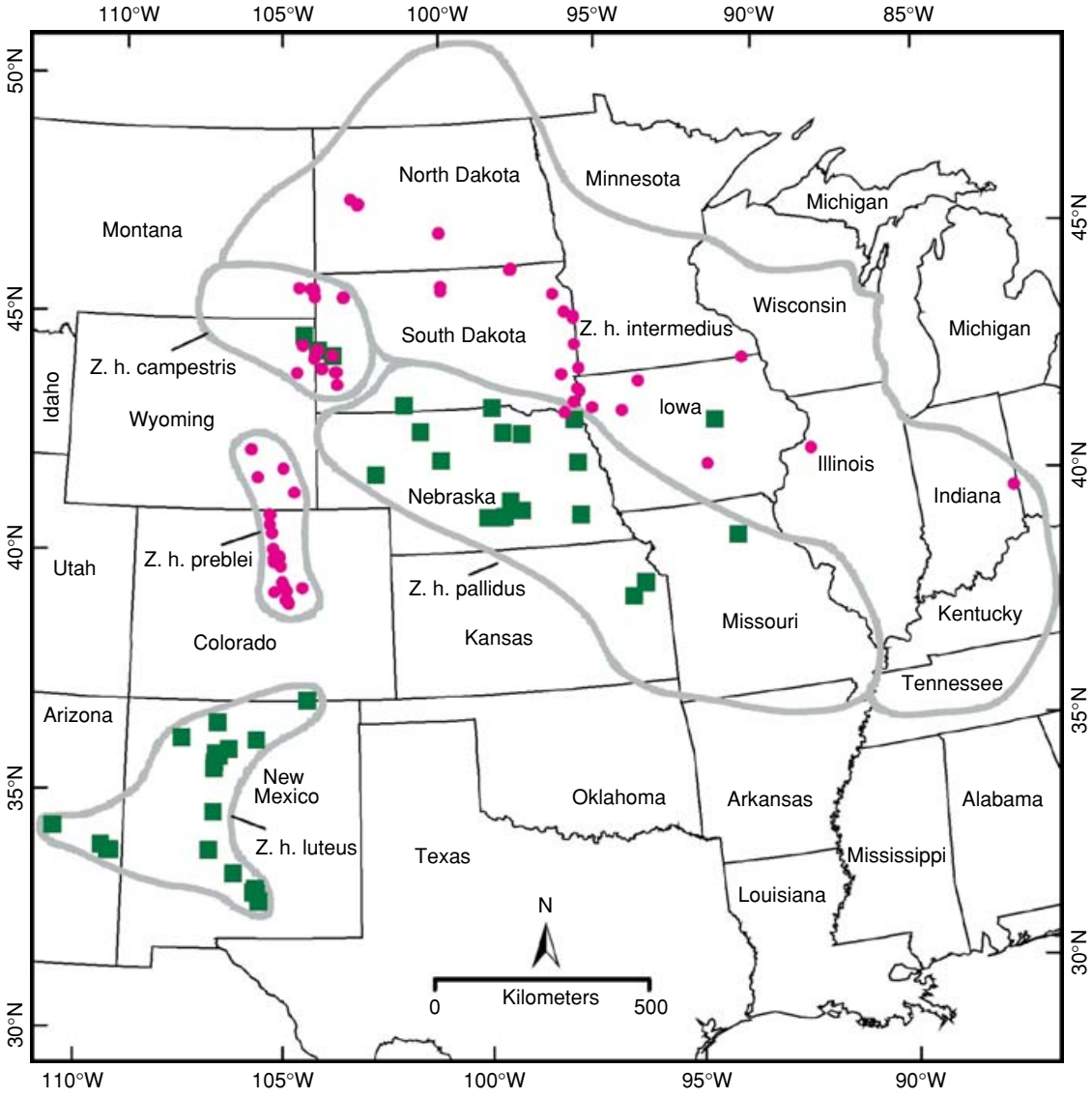

Fig. 4. Map showing collection locations of specimens used in mtDNA analyses. Multiple samples were taken from some locations. Grey outlines indicate subspecies ranges (Krutzsch, 1954; Hafner *et al*., 1981). Pink circles indicate specimens on the *Z. h. preblei*/*Z. h. campestris* mtDNA lineage, green squares indicate specimens on the *Z. h. luteus*/*Z. h. pallidus* mtDNA lineage.

or few, individuals per site across a broad geographical area (Hartl & Clark, 1999). This is supported by the result that when *Z. h. preblei* and *Z. h. intermedius* were further subdivided in biogeographically meaningful ways for analysis, the number of loci violating conditions of Hardy–Weinberg equilibrium dropped sharply (Table 3). Non-random mating (inbreeding) or extensive substructuring in local populations could also potentially contribute to heterozygote deficiency (Wilson, Naish & Boulding, 1999; Yu, Liao & Kao, 2001). A low rate of missing data (2%) suggests that null alleles and allelic drop-out were not likely explanations.

Allelic richness estimates based on putative subspecies designations showed that *Z. h. preblei* had much lower allelic richness than any of the other putative subspecies, suggestive of a strong bottleneck, founder effect, or low effective population size (Table 3). For analyses based on the seven populations, the northern and southern populations of *Z. h. preblei* both had lower allelic richness than any of the remaining subpopulations.

AMOVA tests of the five putative subspecies showed that only 7.5% of the variance was between populations, while 92.5% of the variance was within populations. For the seven population division, only 8.9% of the variance was between populations, while 91.1% of the variance was within populations. When *Z. h. preblei* and *Z. h. campestris* were compared using AMOVA (which provides an estimate of F_{ST} using pairwise distances among alleles), 9.0% of the variance was between populations and 91.0% was within populations.

Three unique alleles were found in *Z. h. preblei* in three loci and these were all at low frequency (<0.05). (The locus dropped because of strong heterozygote deficiency

Table 3. Genetic variability estimates for microsatellite loci used in this study

Comparison	*N*	H_o	H_E	Loci not in *HE*	Private alleles	*A*	F_{IS}
Z. h. preblei	54	0.58	0.74	20, 7	3	6.89	0.212
(*Z. h. preblei* – South)	(33)	0.62	0.69	–	2	(4.49)	(0.087)
(*Z. h. preblei* – North)	(21)	0.51	0.73	–	1	(4.46)	(0.288)
Z. h. campestris	29	0.52	0.78	7, 26	2	9.28 (6.18)	0.333
Z. h. intermedius	46	0.66	0.83	20, 48, 52, 26	8	11.69	0.209
(*Z. h. intermedius* – West)	(38)	0.67	0.81	–	4	(6.75)	(0.159)
(*Z. h. intermedius* – East)	(8)	0.58	0.94	52	4	(9.00)	(0.385)
Z. h. pallidus	34	0.74	0.89	26	7	13.42 (8.58)	0.174
Z. h. luteus	32	0.68	0.85	20, 26	2	10.21 (7.00)	0.189

Allelic richness (*A*) is averaged across loci; *A* values were sub-sampled with FSTAT using a minimum sample size of 29, *A* values in parentheses were sub-sampled using a minimum sample size of 8. Hardy–Weinberg Equilibrium (*HE*) tests were preformed using GENEPOP for five and seven subpopulations and were corrected for multiple comparisons. The mean frequency of private alleles was 0.029 (range 0.013–0.125), as calculated by GENEPOP (Raymond & Rousset, 1995).

Table 4. Maximum likelihood (MDIV) estimates of very recent gene flow between populations of *Zapus hudsonius*

Comparison	*theta*	N_e	*m* (range)	*M* (range)
Z. h. preblei – Z. h. campestris	2.7	27,409	3.3×10^{-6}–3.2×10^{-5}	0.18–1.74
Z. h. campestris – Z. h. intermedius	23.0	230,924	1.3×10^{-6}–1.3×10^{-5}	0.58–5.86
Z. h. pallidus – Z. h. intermedius	10.5	105,622	1.9×10^{-7}–2.3×10^{-6}	0.04–0.48
Z. h. preblei – Z. h. luteus	5.6	56,124	0–1.0×10^{-6}	0.0–0.14
Z. h. preblei – Z. h. pallidus	6.4	64,558	0–2.2×10^{-6}	0.0–0.28
Z. h. preblei – Z. h. intermedius	19.1	191,767	0–$2. 2 \times 10^{-6}$	0.0–0.84

$Theta = 4N_e\mu$, N_e is the estimated effective population size, *m* is the migration rate between populations and *M* is the scaled migration rate. The range of *m* was defined as within 2 Akaike's Information Criterion (AIC) units of the most likely parameter value.

had one private allele at a frequency of 0.55 in the southern population of *Z. h. preblei* and 0.048 in the northern population.) On the basis of these microsatellite analyses, we again reject the hypothesis of uniqueness for *Z. h. preblei* relative to *Z. h. campestris*.

Testing genetic exchangeability

After correcting for N_e, the range of migration estimates (using MDIV) between *Z. h. preblei – Z. h. luteus*, *Z. h. preblei – Z. h. pallidus* and *Z. h. preblei – Z. h. intermedius* included zero, suggesting that little or no very recent mtDNA gene flow has occurred between *Z. h. preblei* and these other subspecies. *Z. h. preblei* and *Z. h. campestris* showed low, but non-zero, levels of very recent gene flow (*m* and *M*) (Table 4). Thus, the null hypothesis of no very recent gene flow between these putative subspecies can be rejected. Gene flow between *Z. h. campestris* and *Z. h. intermedius* was also greater than zero, therefore the null hypothesis of no recent gene flow can also be rejected for those putative subspecies. The null hypothesis of historic genetic exchangeability cannot be rejected using the results of the subspecies tests above.

For microsatellite data, Nei's genetic distance between the seven subpopulations reflected a general pattern of gene flow between adjacent subpopulations that is consistent with isolation by distance (Table 5, Fig. 5): more distant comparisons had larger *D* values, as expected. An exception to this pattern was the high *D* value between *Z. h. luteus* and the southern population of *Z. h. preblei*. While both of these populations showed evidence of gene flow with *Z. h. pallidus* to the east, high genetic distance suggests little or no current gene flow between them. Notably, *D* between the northern and southern populations of *Z. h. preblei* was comparable with *D* between the northern *Z. h. preblei* and *Z. h. pallidus* populations (Fig. 5). Pairwise F_{ST} estimates between *Z. h. preblei* populations (0.10) and adjacent subspecies (0.07–0.16) suggest that the number of migrants per generation is above the cutoff ($Nm < 1$) suggested by Crandall *et al.* (2000) as evidence for rejecting the hypothesis of recent genetic exchangeability, where $F_{ST} = 1/(1 + 4\,Nm)$.

BAPS population clustering suggested a greater degree of genetic structure of the southwestern populations: both the north and south populations of *Z. h. preblei* and *Z. h. luteus* had a high posterior probability (> 0.95) of different allele frequencies, while *Z. h. campestris*, both the eastern

Table 5. Matrix of pairwise genetic distances (Nei's *D*) as calculated by BAPS (Corander *et al*., 2003) above the diagonal and pairwise F_{ST} values (Weir & Cockerham, 1984) as calculated by GENEPOP (Raymond & Rousset, 1995) below the diagonal

Comparison	*preblei* North	*preblei* South	*campestris*	*intermedius* West	*intermedius* East	*pallidus*	*luteus*
preblei – North	–	0.36	0.53	0.49	0.87	0.40	0.66
preblei – South	0.10	–	0.46	0.48	0.90	0.48	0.96
campestris	0.11	0.11	–	0.11	0.43	0.49	0.86
intermedius – West	0.10	0.11	0.01	–	0.43	0.38	0.84
intermedius – East	0.11	0.14	0.03	0.03	–	0.47	0.59
pallidus	0.07	0.10	0.07	0.05	0.01	–	0.34
luteus	0.11	0.16	0.11	0.10	0.03	0.03	–

All pairwise F_{ST} values were significant at $P < 0.05$.

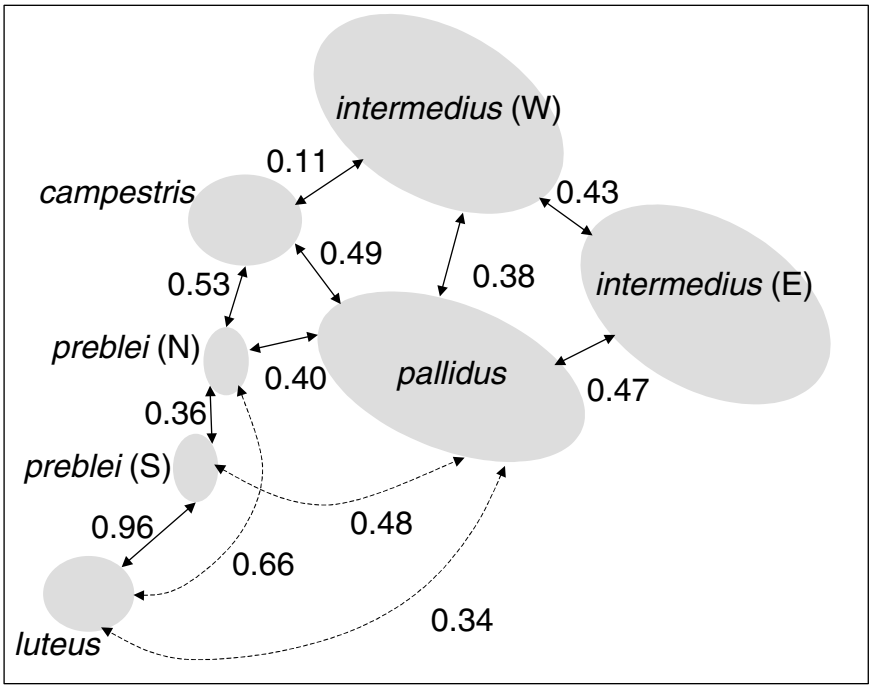

Fig. 5. Schematic diagram of seven subpopulations of *Z. hudsonius* showing Nei's genetic distances (*D*) between subpopulations as determined from five microsatellite loci. Comparisons between non-adjacent subpopulations are denoted by dotted arrows; comparisons between adjacent subpopulations are denoted by arrows. N, north, S, south, E, east, W, west.

and western populations of *Z. h. intermedius* and *Z. h. pallidus* were indistinguishable at this level of posterior probability.

STRUCTURE analyses indicated that $k = 8$ was the most likely cluster number and this was used for assignment analyses, but the variability of the likelihood estimates was high, suggesting that clusters were poorly defined. This result is typical of situations reflecting isolation by distance (Worley *et al*., 2004). STRUCTURE analyses supported a potential, very recent biogeographical separation of northern and southern populations of *Z. h. preblei*. Most individuals in each of these populations were assigned to unique clusters, although 24% of the northern samples were assigned to the cluster 5 (to which all but two of the southern samples were assigned; Table 6). Overall, our analyses suggested a pattern of greater genetic structure in the southwestern populations of *Z. hudsonius*: average values of q_{MAX} were highest in the southern population of *Z. h. preblei*, followed by the northern population of *Z. h. preblei*, then *Z. h. luteus* (Table 7). Likewise, roughly 55% of individuals were assignable at $q > 0.90$ to the southern population of *Z. h. preblei*, whereas the northern population of *Z. h. preblei* had 42% and *Z. h. luteus* had 22% of individuals assignable at this level of q (Table 7). Other groups had few or no individuals assignable at $q > 0.90$ (Table 7). Thus, we could reliably assign only 55% of individuals to the most clearly defined population (the southern population of *Z. h. preblei*). We therefore cannot reject the hypothesis of *recent* genetic exchangeability, or the null hypothesis of *historic* genetic exchangeability (using results of the subspecies tests on mtDNA or microsatellite data).

Testing ecological exchangeability

There is no published evidence of adaptive differences (e.g. selection for cryptic pelage on different rocky

Table 6. Results of STRUCTURE individual-level clustering, with proportion of each subpopulation assigned to each of $k = 8$ clusters, on the basis of the highest value of q (no cut-off value of q was used)

Subpopulation	1	2	3	4	5	6	7	8
Z. h. preblei (North)		0.71	0.05		0.24			
Z. h. preblei (South)		0.03			0.94	0.03		
Z. h. campestris	0.03	0.07	0.03	0.03	0.1	0.17		0.55
Z. h. pallidus	0.21		0.32	0.09		0.03	0.32	0.03
Z. h. luteus	0.44		0.22	0.31			0.03	
Z. h. intermedius (East)	0.25		0.38		0.13		0.13	0.13
Z. h. intermedius (West)	0.03	0.03	0.05	0.05	0.03	0.34	0.03	0.45

'Zero' values are omitted for clarity.

Table 7. Results of STRUCTURE analyses of seven sub-populations of *Zapus hudsonius*, for $k = 8$ clusters, reflecting the trend of greater genetic structure at the south-western extent of the range

Sub-Population	Average value of q_{MAX}	Percentage (and No.) of samples assigned at $q > 0.90$
Z. h. luteus	0.67	21.9% (7)
Z. h. preblei (South)	0.86	54.5% (18)
Z. h. preblei (North)	0.85	42.9% (9)
Z. h. campestris	0.61	0
Z. h. pallidus	0.47	0
Z. h. intermedius (West)	0.64	2.6% (1)
Z. h. intermedius (East)	0.44	0

Sub-populations are listed generally from south to north and from west to east.

substrates as found by Hoekstra & Nachman, 2003) or ecological differences (e.g. major habitat and/or climatic differences) that would be expected to result in notable adaptive differences between *Z. h. preblei* and other adjacent subspecies (Krutzsch, 1954; Whitaker, 1972, 1999; Jones 1981; Clark & Stromberg, 1987; see Cryan, 2004 for an in-depth review). These animals live in a range of similar habitat types and appear to have similar life histories. While the absence of evidence does not necessarily mean there is evidence of absence, there do not appear to be any adaptive differences that prevent the *Z. hudsonius* subspecies in this study from being ecologically exchangeable. We therefore cannot reject the null hypothesis of historic or recent ecological exchangeability.

While PCA and LDA on cranial measurements have limitations for inferring adaptive divergence, there appears to be almost no difference between *Z. h. preblei* and *Z. h. campestris* from a multivariate perspective. While *Z. h. intermedius* appears to be smaller than *Z. h. preblei* and *Z. h. campestris*, there is substantial overlap and no reliable multivariate distinguishability using the more powerful LDA. Because size alone (represented by PC1 in PCA) can be due to ecophenotypic and/or genetic differences, these results do not provide an adequate basis for rejecting the null hypothesis of ecological exchangeability.

DISCUSSION

Putative subspecies and taxonomic conclusions

Our morphometric results refuted the univariate quantitative basis for the description of *Z. h. preblei* as a subspecies. Distinguishability between groups is the key to valid systematic divisions, which for morphometric data is a multivariate question that should be investigated as such. Our multivariate analyses also refuted the distinguishability of *Z. h. preblei*, as well as *Z. h. intermedius*. We found that microsatellite and mtDNA analyses also did not support *Z. h. preblei* as a separate subspecies. *Zapus h. preblei* appears instead to be a population of *Z. h. campestris* with lower genetic variability. If *Z. h. preblei* had evolved in long-term isolation from *Z. h. campestris*, it should at least approach reciprocal monophyly of mtDNA with strong bootstrap support. This was not the case and the amount of molecular variance found between populations was below that required in our critical tests. The same conclusion was found for *Z. h. intermedius*. Additional sequence data would undoubtedly reveal additional structure, but would be unlikely to change the basic conclusions.

Although there are limitations to the applicability of microsatellites to phylogeographic questions (Paetkau *et al*., 1997; Balloux *et al*., 2000; Zink, 2004), analysis of microsatellite data also leads us to reject the hypothesis of uniqueness for *Z. h. preblei*, *Z. h. campestris* and *Z. h. intermedius*. These results were concordant with those obtained from morphometrics and mtDNA, except that *Z. h. pallidus* is largely fixed for one lineage of mtDNA relative to adjacent populations of *Z. h. campestris* and *Z. h. intermedius* and it shows low levels of differentiation for microsatellite loci (Table 5, Fig. 5). Lineage sorting is one possible explanation for the greater genetic structure in mtDNA among these subspecies. Sex-biased dispersal, with males moving nuclear genetic material over longer distances, is also a possibility. A selective sweep appears to have been ruled out by neutrality tests.

Based on hypothesis testing using four lines of evi–dence – morphometrics, mtDNA, microsatellites and a lack of recognised adaptive differences – we synonymise *Z. h. preblei* and *Z. h. intermedius* with *Z. h. campestris*, which was described first as the prairie jumping mouse by Preble (1899). Because we did not analyse cranial

morphometric data for *Z. h. luteus* and *Z. h. pallidus*, we are cautious about their taxonomic status at this time. However, our preliminary results are consistent with Jones' (1981) findings that there do not appear to be any recognisable subspecies of *Z. hudsonius* in the study area.

Testing genetic and ecological exchangeability

Assignment test results reflected a general pattern of gene flow between populations, with lower gene flow to isolated populations at the margins of the range. While *Z. h. preblei* had a higher proportion of individuals assignable at high confidence ($q > 0.90$) in STRUCTURE analyses (Table 7), both as a combined group or split into northern and southern populations, *Z. h. preblei* also showed much lower allelic richness than the other groups (Table 3). This implies that the genetic structure observed in the BAPS and STRUCTURE analyses for this region may stem from repeated population bottlenecks or founder effects and recent isolation, which reduced the microsatellite alleles to a subset of those present in neighbouring populations, rather than long-term divergence. This interpretation is consistent with mtDNA analyses, which show fewer haplotypes and lower nucleotide diversity in *Z. h. preblei*.

Estimates of *D* imply that gene flow between the northern population of *Z. h. preblei* and the adjoining populations of *Z. h. pallidus* and *Z. h. campestris* has occurred more recently than between *Z. h. preblei* and *Z. h. luteus* and that the level of isolation between the northern and southern populations of *Z. h. preblei* is comparable with that between the northern population of *Z. h. preblei* and *Z. h. pallidus*, as well as between the eastern and western populations of *Z. h. intermedius*. Isolation of southwestern populations of *Z. hudsonius* therefore appears to be a recent phenomenon that has accompanied the Holocene drying of the Great Plains as well as more recent agriculture and development (Hafner *et al*., 1981; Jones, 1981). Population densities of *Z. hudsonius* are limited by competition with *Microtus* (Boonstra & Hoyle, 1986) as well as by anthropogenic causes.

Although some degree of population discrimination can be achieved for *Z. h. preblei* using discriminant analysis and assignment tests, classification of individuals to this putative subspecies with a high degree of confidence (as determined by posterior probabilities) is low. While our ability to quantitatively assess ecological exchangeability was limited, as is often the case (Crandall *et al*., 2000), the morphometric analyses address at least some aspects of ecological interchangeability. In lieu of better options, such analyses can provide evidence suggestive of consistent physical differences that may be attributable to different selective environments.

In summary, we found no convincing evidence that would result in our rejection of the hypotheses of genetic or ecological exchangeability on recent or historic timescales for *Z. h. preblei*, *Z. h. campestris* and *Z. h. intermedius*. Therefore, these putative subspecies do not appear to be distinct populations (Crandall *et al*., 2000). The results are consistent with the fact that *Z. h. preblei*, in particular the southern population, is a peripheral population at the edge of the species range and subject to founder effects. *Zapus h. luteus* does not appear to have had much current or historic gene flow with *Z. h. preblei* based on mtDNA and microsatellite analyses. The extent to which very recent human development (e.g. the past 100 years) may have contributed to additional isolation and bottlenecks is unknown. Both of these would be expected to increase the degree of genetic distance from other populations (Hedrick, Gutierrez-Espeleta & Lee, 2001). Regardless of whether more relaxed criteria are used for testing recent genetic exchangeability, or if trapping studies confirm isolation, a rejection of *recent* genetic exchangeability would be insufficient to treat *Z. h. preblei* as a distinct population using the criteria proposed by Crandall *et al*. (2000). The results also suggest that *Z. hudsonius* from healthy nearby populations could be used to augment or re-establish populations within the range of *Z. h. preblei*, should this become a management objective.

Although there may be genetically-based differences that are currently unknown, the majority of the evidence suggests that neutral genetic divergence among these putative subspecies is low and adaptive genetic divergence is non-existent. Therefore, based on the evidence examined here, *Z. h. preblei* does not appear to qualify as a distinct population using the approach of Crandall *et al*. (2000).

Currently, the US-ESA requires that a Distinct Vertebrate Population Segment (DPS) be 'discrete' and 'of significance' (US Fish and Wildlife Service, US National Oceanic and Atmospheric Administration, 1996). Discrete is defined as 'markedly separated from other populations of the same taxon by physical, physiological, ecological, or behavioral factors' using evidence from 'quantitative measures of genetic or morphological discontinuity' (US Fish and Wildlife Service, US National Oceanic and Atmospheric Administration, 1996). Significance is defined as 'evidence that loss of the discrete population segment would result in a significant gap in the range of a taxon.' While both of these criteria are vague, our results for *Z. h. preblei* and its neighbouring populations do not appear to support the discrete requirement and the broad distribution of *Z. hudsonius* does not appear to support the significance requirement.

Evaluating the genetic basis of taxa and populations proposed for listing or delisting under the US-ESA

Two types of error are inherent in the process of listing taxa or populations as endangered or threatened and both can have negative effects on conservation (National Research Council, 1995). The first, as illustrated by *Z. h. preblei*, occurs where an invalid taxon or non-distinct population is listed. This affects other species because limited conservation resources are then misallocated. It can also have negative socioeconomic consequences, including the restriction of some benign human activities and can undermine public support for the US-ESA. The other type of error occurs when a valid taxon is not listed because its unique properties were not identified and it goes extinct – an irreversible loss of biodiversity. Like

Type I and II statistical errors, criteria set relative to one of the ESA listing errors will influence the rate of the other type of error. Well-defined criteria and regulations are needed for US-ESA listing procedures that minimise both errors to the maximum extent possible.

Criteria for genetic uniqueness need to adequately identify natural discontinuities in gene pool variation and distinguish these from recent (e.g. last 100 years) differences that may be due to genetic drift from human-induced population bottlenecks or isolation (Hedrick *et al*., 2001; Brown *et al*., 2004). These criteria should not be so stringent that unique organisms fail to be listed.

Recognising the problem of using only genetic data, Crandall *et al*. (2000) proposed that populations be recognised as distinct if they show evidence of recent genetic isolation (not genetically exchangeable) and adaptive differences (not ecologically exchangeable), or both historic and recent adaptation (not ecologically exchangeable). However, these authors did not fully address the question of how much genetic difference is sufficient for each of these distinctions.

In our study, we used a three-step approach to test the validity of subspecies and the validity of distinct populations. This process could be reduced to two steps if candidates for listing met a minimum standard of genetic uniqueness within the conceptual framework of Crandall *et al*. (2000). First, test the original taxonomic or DPS description. This is especially important below the level of species, because original descriptions often relied on poorly-quantified traits that have an unknown genetic basis (Hendry *et al*., 2000; Wehausen & Ramey, 2000; Zink, 2004). Second, apply critical tests (like the ones used in this study) to the hypotheses of genetic and ecological exchangeability as proposed by Crandall *et al*. (2000). Establishing a conceptually sound and consistent methodological approach for listings is imperative because there are currently no uniform criteria among taxonomic groups (or investigators) as to what constitutes a species, subspecies, or DPS (Avise & Johns, 1999; Crandall *et al*., 2000). This approach applies equally to taxa being considered for listing or delisting under the US-ESA and could also be applied to biodiversity laws in other countries. Because 561 out of the 1855 species listed under the US-ESA occur outside the USA, the basis of US-ESA listings is also an international scientific issue.

Acknowledgements

We thank the KU, MSB, NSM and DMNS for access to zoological and genomic research collections. Pioneer Environmental Services and the City of Fort Collins provided two ear punch specimens. T. Quinn and S. Oyler-McCance provided access to the DNA sequencing facility at Rocky Mountain Center for Conservation Genetics and Systematics. We thank four anonymous reviewers and P. Krutzsch for useful comments and. L. M. Brown for keen editing. Funding was provided by the State of Wyoming, U.S. Fish & Wildlife Service, Department of Energy, Department of Defense, U.S. Forest Service and the DMNS.

REFERENCES

Avise, J. C. & R. M. Ball Jr. (1990). Principles of genealogical concordance in species concepts and biological taxonomy. *Oxf Surv Evol Biol* **7**: 45–67.

Avise, J. C. & Johns, G. C. (1999). Proposal for a standardized temporal scheme of biological classification for extant species. *Proc. Natl. Acad. Sci. USA* **96**: 7358–7363.

Ball, M. R. & Avise, J. C. (1992). Mitochondrial DNA phylogeographic differentiation among avian populations and the evolutionary significance of subspecies. *Auk* **109**: 626–636.

Balloux, F., Brunner, H., Lugon-Moulin, N., Hausser, J. & Goudet, J. (2000). Microsatellites can be misleading: an empirical and simulation study. *Evolution* **54**: 1414–1422.

Boonstra, R. & Hoyle, J. A. (1986). Rarity and coexistence of a small hibernator, *Zapus hudsonius*, with fluctuating populations of *Microtus pennsylvanicus* in the grasslands of southern Ontario. *J. Anim. Ecol.* **55**: 773–784.

Brown, L. M., Ramey, R. R., Tamburini, B. & Gavin, T. A. (2004). Population structure and mitochondrial DNA variation in sedentary Neotropical birds isolated by forest fragmentation. *Conserv. Genet.* **5**: 743–757.

Burnham, K. P. & Anderson, D. R. (1998). *Model selection and inference.* New York: Springer-Verlag.

Clark, T. W. & Stromberg, M. R. (1987). *Mammals in Wyoming.* Lawrence, KA: University of Kansas Museum.

Conner, M. M. & Shenk, T. M. (2003). Distinguishing *Zapus hudsonius preblei* from *Zapus princeps princeps* by using repeated cranial measurements. *J. Mamm.* **84**: 1456–1463.

Corander, J., Waldmann, P. & Sillanpaa, M. J. (2003). Bayesian analysis of genetic differentiation between populations. *Genetics* **163**: 367–374.

Crandall, K. A., Bininda-Emonds, O. R. P., Mace, G. M. & Wayne, R. K. (2000). Considering evolutionary processes in conservation biology. *Trends Ecol. Evol.* **15**: 290–295.

Cronin, M. A. (1997). Systematics, taxonomy and the Endangered Species Act: the example of the California gnatcatcher (*Polioptila californica*). *Wildl. Soc. Bull*. **25**: 661–666.

Cryan, P. M. (2004). *Synthesis of existing information on meadow jumping mice* (***Zapus hudsonius***) *in the northern Great Plains*. An information review compiled for the U.S. Fish and Wildlife Service, Region 6, Denver, Colorado. USGS Fort Collins Science Center, 2150 Centre Ave., Building C, Fort Collins, CO 80526.27 pp.

Excoffier, L., Smouse, P. E. & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial restriction data. *Genetics* **131**: 479–491.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fraser, D. J. & Bernatchez, L. (2001). Adaptive evolutionary conservation: towards a unified concept for defining conservation units. *Mol. Ecol.* **10**: 2741–2752.

Goldstein, P. Z., DeSalle, R., Amato, G. & Vogler, A. P. (2000). Conservation genetics at the species boundary. *Conserv. Biol.* **14**: 120–131.

Gordon, R. E., Lacy, J. K. & Streeter, J. R. (1997). Conservation under the Endangered Species Act. *Environ. Int.* **23**: 359–419.

Goudet, J. (1995). FSTAT (Version 1.2): a computer program to calculate F-statistics. *J. Heredity* **86**: 485–486.

Hafner, D. J., Petersen, K. E. & Yates, T. L. (1981). Evolutionary relationships of jumping mice (Genus *Zapus*) of the southwestern United States. *J. Mamm.* **62**: 501–512.

Hartl, D. L. & Clark, A. G. (1989). *Principles of population genetics.* Sunderland, MA: Sinauer Assoc.

Hedrick, P. W., Gutierrez-Espeleta, G. A. & Lee, R. N. (2001). Founder effect in an island population of bighorn sheep. *Mol. Ecol.* **10**: 851–857.

Hendry, A. P., Vamosi, S. M., Latham, S. J., Heibuth, J. C. & Day, T. (2000). Questioning species realities. *Conserv. Gen.* **1**: 67–76.

Hoekstra, H. E. & Nachman, M. W. (2003). Different genes underlie adaptive melanism in different populations of rock mice. *Mol. Ecol.* **12**: 1185–1194.

Huson, D. H. (1998). SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14**: 68–73.

Jones, G.S. (1981). *The systematics and biology of the genus **Zapus** (Mammalia, Rodentia, Zapodidae)*. Unpublished Ph.D. thesis: Indiana State University, Terre Haute.

Keita, S. O. Y., Kittles, R. A., Royal, C. D., Bonney, G. E., Furbert-Harris, P., Dunston, G. M. & Rotimi, C. N. (2004). Conceptualizing human variation. *Nature Genet. Suppl.* **36**:17–20.

Krutzsch, P. H. (1954). North American jumping mice (genus ***Zapus***). *Univ. Kansas Publ., Mus. Nat. Hist.* **4**: 349–472.

Kumar, S., Koichiro, K., Jakobsen, I. & Nei, M. (2002). *MEGA: molecular evolutionary genetics analysis, Version 2.0.* Pennsylvania State University, University Park and Arizona State University, Tempe.

Leberg, P. L. (2002). Estimating allelic richness: effects of sample size and bottlenecks. *Mol. Ecol.* **11**: 2445–2449.

Matson, C. W. & Baker, R. J. (2001). DNA sequence variation in the mitochondrial control region of red-backed voles (*Clethrionomys*). *Mol. Biol. Evol.* **18**: 1494–1501.

National Research Council. (1995). *Science and the Endangered Species Act.* Washington, DC: National Academy Press.

Nielsen, R. & Wakeley, J. (2001). Distinguishing migration from isolation: a Markov chain Monte Carlo approach. *Genetics* **158**: 885–896.

Oyler-McCance, S. J., St. John, J., Parker, J. M. & Anderson, S. H. (2005). Characterization of microsatellite loci isolated in midget-faded rattlesnakes (*Crotalus viridis concolor*). *Mol. Ecol. Notes* **5**: 455–458.

Paetkau, D., Waits, L. P., Clarkson, P. L., Craighead, L. & Strobeck, C. (1997). An empirical evaluation of genetic distance statistics using microsatellite data from bear (Ursidae) populations. *Genetics* **147**: 1943–1957.

Palneirim, J. M. (1998). Analysis of skull measurements and measurers: can we use data obtained by various observers? *J. Mamm.* **79**: 1021–1028.

Pearse, D. E. & Crandall, K. A. (2004). Beyond Fst: analysis of population genetic data for conservation. *Conserv. Genet.* **5**: 585–602.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Preble, E. A. (1899). Revision of the jumping mice of the genus ***Zapus***. *N. Am. Fauna* **15**: 1–41.

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

Quimby, D. C. (1951). The life history and ecology of the jumping mouse, ***Zapus** hudsonius. Ecol. Monogr.* **21**: 61–95.

Raymond, M. & Rousset, F. (1995). An exact test for population differentiation. *Evolution* **49**: 1280–1283.

Reyment, R. (1990). Reification of classical multivariate statistical analysis in morphometry. In *Proceedings of the Michigan morphometrics workshop*: 123–144.

Reyment, R. A., Blackith, R. E. & Campbell, N. A. (1984). *Multivariate morphometrics.* Orlando, FL: Academic Press.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.

Sokal, R. R. & Rohlf, F. J. (1981). *Biometry*. San Francisco: W. H. Freeman and Company.

Swofford, D. L. (2002). *PAUP*: Phylogenetic Analysis Using Parsimony (and other methods).* Version 4.0b10. Sunderland, MA: Sinauer Associates.

Tavare, S. (1986). Some probabilistic and statistical problems in the analysis of DNA sequences. *Lect. Math. Life Sci.* **17**: 57–86.

US Fish and Wildlife Service, US National Oceanic and Atmospheric Administration (1996). Policy regarding the recognition of distinct population segments under the Endangered Species Act. *The Federal Register* **61**: 4722–4725.

U.S. Fish & Wildlife Service (1998). Final rule to list the Preble's meadow jumping mouse as a threatened species. *The Federal Register* **53**: 26517–26530.

Wehausen, J. D. & Ramey, R. R. (2000). Cranial morphometric and evolutionary relationships in the northern range of *Ovis canadensis*. *J. Mamm.* **81**: 145–161.

Wehausen, J. D., Ramey, R. R. & Epps, C. W. (2004). Experiments in DNA extraction and PCR amplification from bighorn sheep feces: the importance of DNA extraction method. *J. Hered*. **95**: 503–509.

Weir, B. S. & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.

Whitaker, J. O. (1972). ***Zapus** hudsonius*. *Mamm. Spec.* **11**: 1–7.

Whitaker, J. O. (1999). Meadow jumping mouse (*Zapus hudsonius*). In *The Smithsonian book of North American Mammals*: 666–667. Wilson, D. E. & Ruff, S. (eds). Washington, DC: Smithsonian Institution Press.

Wilson, E. O. & Brown, W. L. (1953). The subspecies concept and its taxonomic applications. *Syst. Zool.* **2**: 97–122.

Wilson, A. B., Naish, K.-A. & Boulding, E. G. (1999). Multiple dispersal strategies of the invasive quagga mussel (*Dreissena bugensis*) as revealed by microsatellite analysis. *Can. J. Fish. Aquat. Sci.* **56**: 2248–2261.

Worley, K., Strobeck, C., Arthur, S., Carey, J., Schwantje, H., Veitch, A. & Coltman, D. W. (2004). Population genetic structure of North American thinhorn sheep (*Ovis dalli*). *Mol. Ecol.* **13**: 2545–2556.

Wright, S. (1921). Systems of mating. *Genetics* **6**: 111–178.

Yu, H., Liao, Y. & Kao, C. (2001). Relatedness structure and individual identification in a semi-fossorial shrew (Soricidae: *Anourosorex squamipes*) – an application of microsatellite DNA. *Zool. Stud.* **40**: 226–232.

Zink, R. M. (2004). The role of subspecies in obscuring avian biological diversity and misleading conservation policy. *Proc. Roy. Soc. Lond.* **271**: 561–564.

APPENDIX 1. Catalog numbers of specimens used for cranial measurements.

Specimens are listed in the order they were examined. DMNS, Denver Museum of Nature & Science; KU, University of Kansas Museum of Natural History.

DMNS: *Z. h. preblei* 9572, 9864, 10380, 9843, 9853, 9570, 9569, 9562, 9561, 9315, 9205, 9204, 9868, 9862, 10355, 10404, 10269, 10354, 10169, 10265, 10267, 2822, 10604, 9876, 10618, 10630, 10621, 9564, 9312, 10635, 9877, 10620, 10611, 9571, 10266, 10610, 9579, 10613 and 10615.

DMNS: *Z. h. campestris* 8512.

KU: *Z. h.campestris* 101551, 101552, 101554, 101555, 101558, 101560, 87040, 87041, 87042, 87034, 87035, 87036, 87037, 112664, 112657, 20835, 20836, 20837, 20838, 20839, 20840, 20842, 20843, 20844, 20845, 20846, 20847, 20848, 20849, 20851, 20850, 20852, 41450, 41451, 42467, 42468, 42469, 42471, 42517 and 42518.

KU: *Z. h. intermedius* 153184, 153186, 153187, 153188, 153189, 159186, 141254, 141255, 159188, 123023, 123026, 123028, 123029, 123031, 123032, 123033, 108589, 123035, 116266, 116267, 116262, 116263, 116264, 116268, 108068, 116265, 104062, 37275, 154080, 47773, 47774, 47775, 47776, 47777, 47779, 47781 and 47784.

APPENDIX 2. Specimens of *Zapus hudsonius* used in phylogenetic and population genetic analyses.

These are listed by museum or tissue archive catalog number.

DMNH, Denver Museum of Nature & Science; TK, Texas Tech; KU, University of Kansas; UNSM, University of Nebraska State Museum; MSB and NK, Museum of Southwestern Biology; PIONEER, Pioneer Environmental Services.

Abbreviations for states are as follows: AZ, Arizona; CO, Colorado; IL, Illinois; IN, Indiana; IA, Iowa; KS, Kansas; MO, Missouri; MT, Montana; NM, New Mexico; NE, Nebraska; SD, South Dakota; WY, Wyoming. State abbreviations are followed by counties. The location of haplotypes in the table approximately corresponds to the location of the haplotypes in the neighbour-joining tree. Representative individuals used in phylogenetic analysis are indicated with an asterisk

Additional specimens with identical mtDNA haplotype: ID, state and county	Subspecies	Haplotype	Additional specimens with identical mtDNA haplotype: ID, state and county	Subspecies	Haplotype
MSB40951, AZ:Apache*	*Z. h. luteus*	L6	NK871, NM:Otero	*Z. h. luteus*	
MSB40994, AZ:Apache	*Z. h. luteus*		NK884, NM: Socorro	*Z. h. luteus*	
MSB89194, AZ:Navajo*	*Z. h. luteus*	L5	DMNH8630: CO:Las Animas	*Z. h. luteus*	
MSB86344, AZ:Apache*	*Z. h. luteus*	L/PAL/C2	DMNH8631, CO:Las Animas*	*Z. h. luteus*	L3
MSB91627, AZ:Navajo	*Z. h. luteus*		DMNH8632, CO:Las Animas*	*Z. h. luteus*	L2
MSB91675, AZ:Apache	*Z. h. luteus*		DMNH8634, CO:Las Animas	*Z. h. luteus*	
NK1584, AZ:Apache	*Z. h. luteus*		NK9976, NM:Bernalillo*	*Z. h. luteus*	L1
DMNH8635, CO:Las Animas	*Z. h. luteus*		MSB62103, NM:Valencia	*Z. h. luteus*	
DMNH8633, CO:Las Animas	*Z. h. luteus*		MSB58370, NM:Rio Arriba*	*Z. h. luteus*	L/PAL/C1
KU41451, WY:Crook	*Z. h. campestris*		MSB56980, NM:Sandoval	*Z. h. luteus*	
KU153706, KS:Leavenworth	*Z. h. pallidus*		MSB56986, NM:Sandoval	*Z. h. luteus*	
KU112661, SD: Lawrence	*Z. h. campestris*		MSB56987, NM:Sandoval	*Z. h. luteus*	
UNSM20596, NE:Buffalo*	*Z. h. pallidus*	PAL10	MSB56991, NM:Sandoval	*Z. h. luteus*	
UNSM26492, NE:Buffalo*	*Z. h. pallidus*	PAL9	MSB56993, NM:Sandoval	*Z. h. luteus*	
UNSM20879, NE:Buffalo	*Z. h. pallidus*		MSB62096, NM:Sandoval	*Z. h. luteus*	
UNSM13217, NE:Cherry*	*Z. h. pallidus*	PAL8	NK856, NM:Sandavol	*Z. h. luteus*	
UNSM12980, NE:Garden	*Z. h. pallidus*		KU112665, SD:Lawrence	*Z. h. campestris*	
UNSM12991, NE:Garden	*Z. h. pallidus*		KU109963, SD:Lawrence	*Z. h. campestris*	
UNSM26316, NE:Hall	*Z. h. pallidus*		KU110033, SD:Bennett	*Z. h. pallidus*	
UNSM20744, NE:Hall	*Z. h. pallidus*		KU110022, SD:Bennett*	*Z. h. pallidus*	PAL2
UNSM20747, NE:Hall	*Z. h. pallidus*		UNSM27388, SD:Clay*	*Z. h. pallidus*	PAL1/
UNSM26462, NE:Merrick	*Z. h. pallidus*		UNSM27389, SD:Clay	*Z. h. pallidus*	INT-XV
UNSM13067, NE:Thomas	*Z. h. pallidus*		KU116266, IO:Buena Vista	*Z. h. intermedius*	
KU116269, IO:Tama*	*Z. h. intermedius*	INT-XVI	KU140721, SD:Brown	*Z. h. intermedius*	
UNSM17482, NE:Antelope*	*Z. h. pallidus*	PAL7	KU153190, SD:Walworth	*Z. h. intermedius*	
UNSM17495, NE:Antelope	*Z. h. pallidus*		KU153209, SD:Minnehaha	*Z. h. intermedius*	
UNSM17498, NE:Antelope	*Z. h. pallidus*		KU153212, SD:Minnehaha	*Z. h. intermedius*	
UNSM17499, NE:Antelope	*Z. h. pallidus*		KU153221, SD:Moody	*Z. h. intermedius*	
UNSM13084, NE:Dixon	*Z. h. pallidus*		KU147020, SD:Brown*	*Z. h. intermedius*	INT-XIV
UNSM14008, NE:Dodge	*Z. h. pallidus*		KU153176, SD:Brown	*Z. h. intermedius*	
UNSM13118, NE:Holt	*Z. h. pallidus*		KU153177, SD:Brown	*Z. h. intermedius*	
UNSM13343, NE:Lancaster	*Z. h. pallidus*		KU153180, SD:Brown	*Z. h. intermedius*	
UNSM13119, NE:Holt*	*Z. h. pallidus*	PAL6	KU153181, SD:Brown	*Z. h. intermedius*	
UNSM13065, NE:Thomas	*Z. h. pallidus*		KU101564, SD:Pennington*	*Z. h. campestris*	C8/10/
UNSM17727, NE:Boyd*	*Z. h. pallidus*	PAL5	DMNH10638/TK86190, WY:Weston	*Z. h. campestris*	INT-VI
UNSM20600, NE:Buffalo*	*Z. h. pallidus*	PAL4	DMNH10639/TK86191, WY:Weston	*Z. h. campestris*	
KU109633, KS:Osage	*Z. h. pallidus*		KU101558, SD:Pennington	*Z. h. campestris*	
KU109634, KS:Osage	*Z. h. pallidus*		KU123593, MT:Carter	*Z. h. campestris*	
KU153597, MO:Macon*	*Z. h. pallidus*	PAL3	KU123598, MT:Carter	*Z. h. campestris*	
KU153598, MO:Macon	*Z. h. pallidus*		KU123599, MT:Carter	*Z. h. campestris*	
KU153784, KS:Douglas	*Z. h. pallidus*		KU115700, ND:Burleigh	*Z. h. intermedius*	
KU153707, KS:Leavenworth	*Z. h. pallidus*		KU115702, ND:Burleigh	*Z. h. intermedius*	
MSB37154, NM:Otero*	*Z. h. luteus*	L4	KU115710, ND:Burleigh	*Z. h. intermedius*	
MSB61696, NM:Otero	*Z. h. luteus*		KU115731, SD:Walworth	*Z. h. intermedius*	
MSB61684, NM:Otero	*Z. h. luteus*		KU115732, SD:Walworth	*Z. h. intermedius*	
MSB61690, NM:Otero	*Z. h. luteus*		KU120018, ND:Burleigh	*Z. h. intermedius*	
MSB61693, NM:Otero	*Z. h. luteus*		KU120019, ND:Burleigh	*Z. h. intermedius*	
MSB61712, NM:Otero	*Z. h. luteus*		KU123021, ND:Dunn	*Z. h. intermedius*	
MSB58369, NM:Rio Arriba	*Z. h. luteus*		KU123022, ND:Dunn	*Z. h. intermedius*	

APPENDIX 2. Continued

Additional specimens with identical mtDNA haplotype: ID, state and county	Subspecies	Haplotype	Additional specimens with identical mtDNA haplotype: ID, state and county	Subspecies	Haplotype
KU123031, ND:Dunn	*Z. h. intermedius*		DMNH9868/TK86032, CO:Douglas	*Z. h. preblei*	
KU123032, ND:Dunn	*Z. h. intermedius*		DMNH9843/TK86034, CO:Boulder	*Z. h. preblei*	
KU159190, SD:Walworth	*Z. h. intermedius*		DMNH10169/TK86048, CO:Boulder	*Z. h. preblei*	
DMNS7764, ND: Mercer	*Z. h. intermedius*		DMNH10266/TK86080,CO:Douglas	*Z. h. preblei*	
KU123033, ND:Dunn*	*Z. h. intermedius*	INT-I	DMNH10269/TK86083,CO:Douglas	*Z. h. preblei*	
KU112663, SD:Lawrence*	*Z. h. campestris*	C9/INT-VII	DMNH10354/TK86090, CO:Boulder	*Z. h. preblei*	
KU115730, SD:Walworth	*Z. h. intermedius*		DMNH10408/TK86098, WY:Albany	*Z. h. preblei*	
KU20839, WY:Crook*	*Z. h. campestris*	C7	DMNH9564/TK86105, CO:Boulder	*Z. h. preblei*	
KU83559, SD:Harding*	*Z. h. campestris*	C6	DMNH9561/TK86109, CO:Larimer	*Z. h. preblei*	
KU20844, WY:Crook*	*Z. h. campestris*	C5/INT-XIII	DMNH9576/TK86115, CO:Douglas	*Z. h. preblei*	
KU42471, WY:Weston	*Z. h. campestris*		DMNH9574/TK86116, CO:Douglas	*Z. h. preblei*	
KU87040, SD:Harding	*Z. h. campestris*		DMNH10520/TK86124,C̃O:Jefferson	*Z. h. preblei*	
KU83557, SD:Harding	*Z. h. campestris*		DMNH10602/TK86163, CO:Elbert	*Z. h. preblei*	
KU87042, SD:Harding	*Z. h. campestris*		KU110013, SD:Custer	*Z. h. campestris*	
KU112660, SD:Lawrence	*Z. h. campestris*		KU123597, MT:Carter	*Z. h. campestris*	
KU115895, SD:Harding	*Z. h. intermedius*		DMNH9579/XM1166, CO:El Paso*	*Z. h. preblei*	C/P2
KU115896, SD:Harding	*Z. h. intermedius*		DMNH9313/XM875, CO:El Paso	*Z. h. preblei*	
KU115897, SD:Harding	*Z. h. intermedius*		DMNH9315/XM879, CO:El Paso	*Z. h. preblei*	
KU20843, WY:Crook*	*Z. h. campestris*	C4	DMNH10380/TK86093, CO:El Paso	*Z. h. preblei*	
KU109970, SD:Lawrence*	*Z. h. campestris*	C3	DMNH9565/TK86106, CO:El Paso	*Z. h. preblei*	
KU120017, ND:Burleigh*	*Z. h. intermedius*	INT-IX	DMNH9563/TK86107, CO:El Paso	*Z. h. preblei*	
KU42469, WY:Weston*	*Z. h. campestris*	C2	DMNH9566/TK86118, CO:El Paso	*Z. h. preblei*	
KU101552, SD:Pennington*	*Z. h. campestris*	C1	DMNH9573/TK86120, CO:Douglas	*Z. h. preblei*	
KU116263, IO:Emmet*	*Z. h. intermedius*	INT-XII	DMNH9572/TK86121, CO:Douglas	*Z. h. preblei*	
KU116265, IO:Plymouth	*Z. h. intermedius*		DMNH9571/TK86122, CO:Douglas	*Z. h. preblei*	
KU147018, SD:Deuel	*Z. h. intermedius*		DMNH9574/TK86166, CO:El Paso	*Z. h. preblei*	
KU153196, SD:Deuel	*Z. h. intermedius*		DMNH10607/TK86167, CO:El Paso	*Z. h. preblei*	
KU153203, SD:Lincon	*Z. h. intermedius*		KU109978, SD:Custer	*Z. h. campestris*	
KU153201, SD:Deuel*	*Z. h. intermedius*	INT-V	KU123592, MT:Carter	*Z. h. campestris*	
DMNH10614/TK86183,CO:El Paso*	*Z. h. preblei*	C/P4	DMNH10405/TK86095,W̃Y:Albany*	*Z. h. preblei*	C/P1
DMNH10331/TK86088,C̃O:Teller	*Z. h. preblei*		DMNH10258/TK86074,W̃Y:Laramie	*Z. h. preblei*	
DMNH10606/TK86165, CO:El Paso	*Z. h. preblei*		DMNH10270/TK86081, CO:Larimer	*Z. h. preblei*	
DMNH10604/TK86169, CO:El Paso	*Z. h. preblei*		DMNH10404/TK86094, WY:Platte	*Z. h. preblei*	
DMNH10612/TK86170, CO:El Paso	*Z. h. preblei*		DMNH10406/TK86096, WY:Albany	*Z. h. preblei*	
DMNH10605/TK86173, CO:El Paso	*Z. h. preblei*		DMNH10407/TK86097, WY:Albany	*Z. h. preblei*	
DMNH10618/TK86182, CO:El Paso	*Z. h. preblei*		DMNH9568/TK86117, CO:Larimer	*Z. h. preblei*	
DMNH10611/TK86185, CO:El Paso	*Z. h. preblei*		PIONEER9A43, CO: Larimer	*Z. h. preblei*	
DMNH10635/TK86196,C̃O:Douglas	*Z. h. preblei*		PIONEER9B89, CO:Larimer	*Z. h. preblei*	
KU109972, SD:Custer	*Z. h. campestris*		KU109984, SD:Custer	*Z. h. campestris*	
DMNH9204/XM871, CO:Boulder*	*Z. h. preblei*	C/P3	KU109985, SD:Custer	*Z. h. campestris*	
DMNH9205/XM872, CO:Boulder	*Z. h. preblei*		KU104062, IO:Winneshiek*	*Z. h. intermedius*	INT-VIII
DMNH9312/XM874, CO:Gilpin	*Z. h. preblei*		KU116264, IO:Emmet	*Z. h. intermedius*	
DMNH9046/XM876, CO:Boulder	*Z. h. preblei*		KU153229, SD:Union	*Z. h. intermedius*	
DMNH9314/XM877, CO:Boulder	*Z. h. preblei*		KU153203, SD:Lincon	*Z. h. intermedius*	
DMNH9203/TK51406, CO:Jefferson	*Z. h. preblei*		KU140722, SD:Brown*	*Z. h. intermedius*	INT-X
DMNH9880/TK86021, CO:Boulder	*Z. h. preblei*		KU153215, SD:Minnehaha*	*Z. h. intermedius*	INT-XI
DMNH9854/TK86026, CO:Douglas	*Z. h. preblei*		KU153205, SD:Lincon	*Z. h. intermedius*	
DMNH9876/TK86029, CO:Douglas	*Z. h. preblei*		KU127252, IL:Henry*	*Z. h. intermedius*	INT-IV
DMNH9857/TK86030, CO:Douglas	*Z. h. preblei*		KU112830, IN:Wayne*	*Z. h. intermedius*	INT-III
DMNH9865/TK86031, CO:Douglas	*Z. h. preblei*		KU108068, IA:Marion*	*Z. h. intermedius*	INT-II

APPENDIX 3. Specimens of *Zapus princeps* used as outgroups in the phylogenetic analysis and specimens that have an identical mtDNA haplotype or are on the same lineage as the mtDNA haplotypes of representative individuals.

Only the mtDNA haplotypes of the three represenative *Z. princeps* individuals were used in the phylogenetic analysis. Note that some individuals previously identified as *Z. hudsonious* have mtDNA haplotypes that are identical to *Z. princeps*. These individuals were presumed to be misidentified and were excluded from any analyses. Abbreviations are the same as those given in Appendix 2. Representative individuals of *Z. princeps* used in the phylogenetic analysis are indicated with an asterisk

Additional specimens with identical mtDNA haplotype or mtDNA on the same lineage with strong bootstrap support: ID, state and county	Subspecies as per museum tag
DMNH9316, WY:Laramie	*Z. p. princeps*
DMNH10327/TK86085, CO:Teller*	*Z. p. princeps*
DMNH10328/TK86086, CO:Douglas	*Z. p. princeps*
DMNH10330/TK86089, CO:Douglas	*Z. p. princeps*
DMNH10873/TK103545, CO:Conejos	*Z. p. princeps*
DMNH10875/TK103589, CO:Las Animas	*Z. p. princeps*
DMNH10874/TK103593, CO:Las Animas	*Z. p. princeps*
DMNH10257/TK86070, WY:Albany	*Z. h. preblei*
DMNH9567/TK86123, WY:Albany	*Z. h. preblei*
DMNH9569/TK86113, WY:Albany	*Z. h. preblei*
DMNH10698/TK86202, WY:Albany	*Z. h. preblei*
DMNH10274/TK86075, WY:Teton*	*Z. p. utahensis*
DMNH10559/TK86135, WY:Teton	*Z. p. utahensis*
DMNH10535/TK86155, WY:Teton	*Z. p. utahensis*
DMNH10542/TK86175, WY:Teton	*Z. p. utahensis*
DMNH9921/TK86039, WY:Park	*Z. p. idahoensis*
DMNH9923/TK86040, WY:Park	*Z. p. idahoensis*
DMNH9925/TK86041, WY:Park	*Z. p. idahoensis*
KU109994, SD:Custer	*Z. h. campestris*
KU123595, MT:Carter	*Z. h. campestris*
KU30814, KS:Douglas	*Z. h. pallidus*
DMNH9595/TK86112, WY:Fremont*	*Z. p. idahoensis*
DMNH9837/TK86028, WY:Fremont	*Z. p. idahoensis*
DMNH9839/TK86037, WY:Fremont	*Z. p. idahoensis*