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PHYLOGEOGRAPHY OF *MICROTUS LONGICAUDUS* IN THE TECTONICALLY AND GLACIALLY DYNAMIC CENTRAL ROCKY MOUNTAINS

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The central Rocky Mountain region of North America lies at the biogeographic crossroads of the Rocky Mountains, Great Plains, and Great Basin. Here, we examine genetic patterns in an abundant, widely distributed, and ecologically important rodent species, the long-tailed vole (*Microtus longicaudus*), across this climatically and tectonically dynamic landscape. We examine patterns of genetic diversity in relation to the species' overall phylogeographic distribution using mitochondrial cytochrome-*b* sequences from modern, historic, ancient, and published samples. Our analyses reveal extensive genetic diversity in the central Rockies, a lack of population structure, and a lack of concordance between genetic and morphological subspecies distributions. Patterns of genetic variation in late-Holocene and modern populations are similar. We conclude that geographical features such as the Continental Divide, previously isolated glacial cover, and major rivers do not result in persistent genetic structure within the long-tailed vole. We also conclude that the central Rocky Mountain region is a zone of secondary contact for divergent mitochondrial lineages of the long-tailed vole.

Key words: ancient DNA (aDNA), cytochrome *b*, dispersal, Greater Yellowstone Ecosystem, *Microtus longicaudus*, phylogeography, Rocky Mountains

The impacts of Quaternary glacial–interglacial cycles on genetic variation in north temperate mammal species have been extensively documented. Emerging patterns from the literature show that past climatic events have left signatures on the modern distribution of genetic diversity within species (Avise 2000; Brunsfeld et al. 2001; Carstens et al. 2005; Jaarola and Searle 2002; Rowe et al. 2004; Taberlet et al. 1998; Zheng et al. 2003), and that postglacial colonization can create variable patterns of genetic diversity. In areas that were completely glaciated by large ice sheets (Waltari et al. 2007), postglacial recovery generally leads to low levels of within-population genetic diversity because of the colonization founder effect (Hayes and Harrison 1992; Hewitt 2004; Sage and Wolff 1986) followed by rapid population expansion (Dalén et al. 2007; Lessa et al. 2003; Runck and Cook 2005). However, studies from the biogeographically complex Rocky Mountains have revealed high-diversity zones of secondary contact that have arisen since the last glacial maximum (Brunsfeld et al. 2001; Demboski and Sullivan 2003; Good and Sullivan 2001; Hafner et al. 1998; Knowles 2001; Swenson and Howard 2005). The impacts of glacial disturbances on genetic diversity reflect both the geological history of the region and the ecological character of the taxon in question (DeChaine and Martin 2005). On a landscape scale, the effects of deglaciation and climatic warming on the distributions of mammalian species differ in timing and magnitude, according to local conditions (Lessa et al. 2003).

The central Rocky Mountains region is a topographically complex, tectonically active landscape that has been heavily influenced by Quaternary glacial cycles. One of North America's hot spots of modern vertebrate species diversity, the Greater Yellowstone Ecosystem (GYE) is located in the heart of the central Rockies. The GYE refers to a geographic area including parts of Montana, Wyoming, and Idaho, encompassing about 30,000 km^2 centered around the high-elevation Yellowstone Plateau (Bruzgul and Hadly 2007). This central, high-elevation area was glaciated until approximately 14,000 years ago (Gennett and Baker 1986; Pierce

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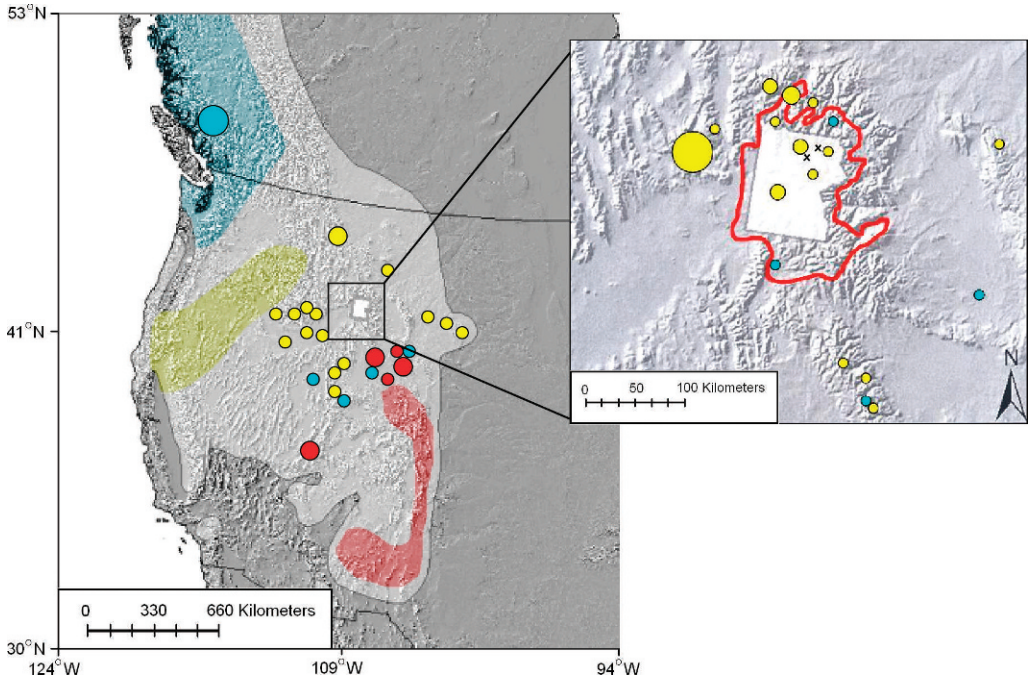


Fig. 1.—Western North America and the southern portion of the range of *Microtus longicaudus*. Circles represent localities sampled for this study. Circle size corresponds to sample size. Colors correspond to haplogroup designations: blue represents individuals from the Northwest Clade; yellow represents individuals from the Central Clade; red represents individuals from the Southern Rockies Clade (see text for a description of the clade designations). The previous sampling across the species range performed by Conroy and Cook (2000a) is represented by the colored areas. Inset shows sampling localities of long-tailed voles in the Greater Yellowstone Ecosystem. Circles represent samples of different sizes at each locality (for details, see appendices): blue represents individuals from the Northwest Clade; yellow represents individuals from the Central Clade. The boundary of the Yellowstone ice cap is shown in red. The fossil localities are designated by ''x'' symbols.

1979; Whitlock and Bartlein 1993; Fig. 1, inset). The ice cap that covered this area was up to 1.6 km thick and 40–80 km wide (Fig. 1, inset). It was an isolated glacial feature, separated by 240 km from the southern edge of the continental ice sheets and surrounded by lower, open, unglaciated terrain (Pierce 1979). Pollen data from the GYE document a rapid colonization of the deglaciated terrain by plants beginning 14,000 years ago. Modern plant communities in the area were established by approximately 4,000 years ago (Gennett and Baker 1986; Whitlock and Bartlein 1993).

Despite the extensive Pleistocene glacial cover throughout this region, Rocky Mountain endemic species persisted in glacial refugia (Brunsfield and Sullivan 2005; Good and Sullivan 2001; Knowles 2001). Information from the distribution of chloroplast lineages of a highly restricted endemic plant (Constance's bittercress [*Cardamine constancei*]) confirms the existence of Pleistocene refugia for plant species in the central Rocky Mountains (Brunsfield and Sullivan 2005). Knowles (2001) and DeChaine and Martin (2005) suggest that repeated glaciation promoted divergence in some alpine insect species. High genetic diversity also has been documented in the endemic red-tailed chipmunk (*Tamias ruficaudus*—Good and Sullivan 2001). In this species, the Bitterroot Range of the central Rockies represents a zone of secondary contact between highly divergent modern subclades. Introgression between the red-tailed chipmunk and another montane chipmunk species also occurs in this region (Good et al. 2003). High genetic diversity and complex phylogeographic patterns also were found in the endemic Uinta ground squirrel (*Spermophilus armatus*—van Tuinen et al. 2008).

In contrast to the attention received by endemic species, phylogenetic investigation of widespread species occurring in the central Rockies has been less frequent. Here, we examine patterns of postglacial genetic diversity and biogeographic connectivity in a wide-ranging generalist, the long-tailed vole (*Microtus longicaudus*). *M. longicaudus* is a small-bodied (<100-g), granivorous rodent with broad habitat tolerances. Long-tailed voles are capable of rapid long-distance dispersal (males have been observed to travel up to 1 km during summer [Jenkins 1948]). Long-tailed voles currently occupy large areas of previously glaciated North America (Hall 1981; Smolen and Keller 1987; Fig. 1) and are among the 1st colonizers of disturbed habitats in montane ecosystems (Hall 1981; Smolen and Keller 1987). The species is found in a variety of habitat types, ranging from closed canopies (spruce–fir forests, aspen groves, willow stands, and alder stands) to open grasslands (Smolen and Keller 1987). The broad physiological tolerances, high rates of reproduction, and generalist ecological niche of the long-tailed vole may contribute to its high local abundance and large geographic range.

The modern geographic range of *M. longicaudus* stretches along the Rocky Mountains from southern Arizona to northern Canada (including areas previously covered by the Laurentide,

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Cordilleran, and isolated Rocky Mountain ice sheets) and extends east to South Dakota and west to the Pacific Ocean (Fig. 1; Hall 1981). Of the 14 described morphological subspecies, only 1 (*M. l. longicaudus*—Smolen and Keller 1987; Tamarin 1985) is found in the central Rocky Mountains, including the GYE. This morphological homogeneity suggests that the central Rocky Mountain region is home to an interbreeding, homogeneous population of long-tailed voles (Hall 1981), distinct morphologically from long-tailed voles in adjacent parts of the range (*M. l. halli* 400 km to the west and *M. l. latus* 400 km to the south [Smolen and Keller 1987; Tamarin 1985]).

A previous phylogeographic investigation of this species (Conroy and Cook 2000a) focused on populations in the Pacific Northwest, but also contained sampling from elsewhere in the species' range, with the notable exception of the central Rocky Mountains. Conroy and Cook (2000a) documented rapid postglacial recolonization of the northwestern portion of the species' range by 1 of 4 allopatric lineages of *M. longicaudus*. They proposed that the deep genetic divergences among these haplogroups reflect the cumulative effects of older Quaternary glacial–interglacial cycles (Conroy and Cook 2000a). Each of the 4 geographically disjunct mitochondrial cytochrome-*b* (*Cytb*) clades (Island, Northwest, Central, and Southern Rockies) was found in a different biogeographic province of western North American (Conroy and Cook 2000a), suggesting that the Continental Divide (dividing eastern and western populations) and the Green River (dividing northern and southern populations) may be historical or current barriers to gene flow, or both. Conroy and Cook (2000a) described the distribution of haplogroups based on their sampling (Fig. 1), but they did not sample the middle of the species' range. The degree of *Cytb* divergence among the 4 lineages (6% uncorrected sequence divergence) was not unusually high for a vole or lemming species (Conroy and Cook 2000a, 2000b; Fedorov et al. 2003; Fink et al. 2004; Galbreath and Cook 2004; Hadly et al. 2004; Jaarola et al. 2004; Jaarola and Searle 2002).

We combined mitochondrial data from recent and subfossil specimens collected in the recently glaciated center of the GYE. The data enable us to describe several thousand years of population dynamics of *M. longicaudus* in the region. Genetic data from recent specimens were collected in order to draw conclusions about past evolutionary processes via phylogenetic inference (Avise 2000). By adding prehistoric population samples, we built an even more complete picture of these historical patterns and processes (Anderson et al. 2005; Hadly et al. 2004). Ancient data may uncover evolutionary patterns that could not have been observed in modern data (Leonard et al. 2000). Alternatively, ancient data may be concordant with modern data, demonstrating temporal stability of population processes (Hadly et al. 1998, 2004; Leonard et al. 2000).

The integration of modern and subfossil data has already contributed to our understanding of complex patterns of genetic variation in other small mammals of the central Rocky Mountains. In a study of the closely related montane vole (*M. montanus*), a combined modern and ancient DNA data set revealed changing patterns of haplotype diversity over time (Hadly et al. 2004). These results were interpreted to reflect past pulses of immigration that would not have been detectable via modern sampling alone. We examined several aspects of the genetic structure of long-tailed voles in the central Rocky Mountains. First, we described phylogeographic patterns within the central (previously unsampled) part of the long-tailed vole's range and related them to the larger phylogeographic structure of the species. Second, we examined genetic diversity in ancient and modern populations of long-tailed voles with respect to population structure and size. Third, we tested the hypothesis that genetic diversity within the GYE arose since the last deglaciation (approximately 14,000 years ago).

We had several expectations based on previous phylogeographic investigations in this region and our knowledge of the target species. The central Rocky Mountains is a biogeographic crossroads that has been patchily disturbed by Pleistocene glaciation. Therefore, we expected that the phylogeography of the long-tailed vole in this region would be complex and variable, as is the case with other local species. The initial phylogeographic description of this species suggests that the Central, Northwest, and Southern Rockies clades that abut the study area may contribute to genetic diversity in the area.

Similarly, we anticipated high genetic diversity in modern populations, paralleling what has been seen in other species in the area (Good and Sullivan 2001). We expected examination of the ancient DNA data to reveal genetic patterns that are concordant with the modern observations, reflecting the operation of similar population processes over millennial timescales. In particular, we expected that frequent, long-distance dispersal that is characteristic of long-tailed voles would result in populations with similar levels of genetic diversity across all sampled time points. Finally, we expected that the genetic divergence of mitochondrial DNAs (mtDNAs) from modern voles in this region would predate the last glacial maximum.

Materials and Methods

Sampling.—We used data from a variety of specimens to thoroughly sample modern and late-Holocene diversity. Our sampling of *M. longicaudus* in the GYE region consisted of 46 modern tissues, 3 specimens from owl pellets, 35 museum skins, and 29 subfossils from the GYE and surrounding areas (Appendices I and II).

Modern tissues.—We collected genetic samples from 46 modern specimens collected with Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, Florida) and 3 specimens from owl pellets in the central Rocky Mountains. We collected specimens from locations within the GYE that were not previously glaciated (Beaverhead County and Gallatin County, Montana; Appendix I), locations in the GYE that were

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previously glaciated (specimens from Teton County, Park County, and Sublette County, Wyoming; and Park County and Beaverhead County, Montana; Appendix I), and from locations in the central Rocky Mountains outside the GYE proper (specimens from Lake County and Fergus County, Montana; Appendix I). Trapping was performed in accordance with guidelines approved by the American Society of Mammalogists (Gannon et al. 2007). Several voles were collected and vouchered at the University of California Museum of Vertebrate Zoology; however, many of the animals were trapped during ongoing ecological experiments (Spaeth 2009) and were therefore not vouchered.

Complete *Cytb* sequences (1,143 base pairs [bp]) were obtained from modern samples by extraction from liver tissue or skin clips and amplification via the previously published primer pairs MVZ05–Micro06 and Arvic07–Vole14 (Hadly et al. 2004). Owl pellet material was amplified and sequenced following the protocols used on the late-Holocene specimens (below). New DNA sequences have been deposited in GenBank under accession numbers EF028803–EF028912.

Museum specimens.—We also included samples of skin clips from museum specimens. These specimens were collected in the central Rocky Mountains ($n = 28$) and northern Rocky Mountains ($n = 7$; Appendix I: specimens listed from the MVZ, CRCM, and KU). These specimens were collected at various times in the 20th and 21st centuries (Appendix I).

Mitochondrial DNA from museum specimens was extracted in the same building that houses our ancient DNA facility, but museum specimens were processed in a separate room with designated reagents, ensuring spatial separation from modern and ancient specimens. Additionally, museum specimens were extracted by researchers who had not worked in the modern laboratory for at least 1 year. Small (<1-cm^2) pieces of skin were clipped from the venter of the museum specimens and the fur was removed with a sterile razor blade. The extraction procedure was performed using a Qiagen DNEasy Tissue Kit (Qiagen, Valencia, California), with overnight incubation at 55°C in a Proteinase-K buffer solution. After extraction, DNA was amplified with primers previously used for ancient specimens of *M. montanus* (see ''*Amplification and authentication of ancient mitochondrial sequences*'' below [Hadly et al. 2004]).

Late-Holocene specimens.—We obtained ancient DNA from teeth identified morphologically as *Microtus* from 2 Holocene, bushy-tailed woodrat (*Neotoma cinerea*) midden sites in the central GYE, Lamar Cave (Fig. 1, inset; Hadly 1996; Porder et al. 2003) and Waterfall Locality (Fig. 1, inset; Porder et al. 2003). Remarkable preservation combined with the well-studied stratigraphy in both deposits allows for genetic sampling of individuals of *Microtus* from well-dated sediment layers.

The collection radius (the area surrounding the paleontological site from which the fossil remains originated) has been calculated for each site based on stable isotope signatures of the fossil remains and local bedrock (Feranec et al. 2007; Porder et al. 2003). The collection radii for Lamar Cave and Waterfall Locality are 7 km and 15 km, respectively (Feranec et al. 2007; Porder et al. 2003). The radii are small, suggesting that the paleontological sites accurately record local community compositions over the last several millennia (Feranec et al. 2007; Porder et al. 2003).

Isolated teeth of *M. longicaudus* are morphologically indistinguishable from the teeth of another locally abundant vole, *M. montanus*. A total of 75 teeth of *Microtus* from 15 levels (dating from 0 to 2,000 years ago [Feranec et al. 2007; Hadly 1996; Porder et al. 2003]) of Lamar Cave were sampled for the *Cytb* extractions. We genetically identified 17 teeth from 6 time intervals from Lamar Cave as *M. longicaudus* (the majority were found to be *M. montanus* [$n = 46$—Hadly et al. 2004]). Fifteen teeth of *Microtus* from 4 time intervals (dating from 0 to 2,400 years ago [Feranec et al. 2007; Hadly 1996; Porder et al. 2003]) from Waterfall Locality were sampled, yielding 12 *M. longicaudus*. To avoid resampling the same individual, all teeth we analyzed from temporal units and surface deposits were right upper 1st molars (Appendix II).

Amplification and authentication of ancient mitochondrial sequences.—Ancient DNA was successfully extracted from teeth ($n = 29$) and amplified according to polymerase chain reaction protocols described by Hadly et al. (2003, 2004), which were designed to prevent contamination of ancient samples. For each ancient DNA specimen, a 312-bp portion of the *Cytb* gene was amplified in 2 or 3 overlapping fragments according to protocols described by Hadly et al. (2004). We targeted a region of *Cytb* gene previously shown to be informative in phylogeographic reconstruction of closely related *Microtus* species (Hadly et al. 2004). We used the following primer pairs: forward primer CLETH 37L (start position: *Cytb* 311) and reverse primer ARVIC 08L (start position: *Cytb* 471); and forward primer MMONT 1 (start position: *Cytb* 452) and reverse primer MMONT 2L (start position: *Cytb* 653). If the 2nd, larger fragment failed to amplify, we obtained shorter fragments using the following internal primers: forward primer MMONT 3 (start position: *Cytb* 485) and reverse primer MMONT 4 (start position: *Cytb* 595). Ancient DNA sequences from both strands were used to assemble 312-bp contigs. Polymerase chain reactions were run on a Gene Amp PCR system 9700 thermal cycler (Perkin-Elmer, Waltham, Massachusetts) using the following program: initial step of 95°C for 10 min; 45 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 30 s; and a final 10-min 72°C extension.

Ancient sequencing was performed on an ABI PRISM 310 sequencer (Perkin-Elmer) in our ancient DNA facility. Modern sequencing reactions were run on an ABI PRISM 377 sequencer at a separate sequencing facility at Stanford University. A full description of our extraction, controls, and sequencing protocols has been previously published (Hadly et al. 2003, 2004).

Confirmation of sequence data was performed via cloning of representative ancient samples ($n = 3$) following the protocol of Hadly et al. (2003). Fifteen to 20 transformed

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colonies per individual were sequenced with M13 primers. Clones were compared to the original sequence obtained for the individual, and the differences among the clones and the original sequence were scored. We checked for the presence of additional, viable mtDNA sequences among the cloned sequences that might indicate contamination of the specimen. We also examined patterns of variation across the codon positions to determine the level of degradation of the specimen (Appendix III).

We checked for the presence of nuclear inserts and DNA damage in all of our sampled individuals by examining patterns of substitution. Specifically, we checked for the accumulation of stop codons, 1st and 2nd position changes that are indicative of nuclear insertion, and an overabundance of A:T pairs indicative of cytosine deamination (Hadly et al. 2003; Hofreiter et al. 2001; Pääbo et al. 2004).

Statistical analysis.—Phylogenetic relationships among sequences were examined through maximum-likelihood analysis in PAUP* 4.0b10 (Swofford 2002), using a neighbor-joining topology as the starting tree, a heuristic search, and tree-bisection-reconnection branch swapping. The best model of molecular evolution under Akaike's information criterion was determined using Modeltest 3.0 (Posada and Crandall 1998). Trees were rooted using the closely related outgroup vole species *M. montanus* and *M. pennsylvanicus* (Conroy and Cook 2000b). Previously published sequences (1 representative of each haplotype identified by Conroy and Cook [2000a]) were used in phylogenetic analyses.

We built a tree based on the entire *Cytb* gene for the subset of voles from our field-trapped specimens and previously published sequences. This expansive data set enabled us to ascertain more precisely the degree of monophyly and geographic reach of the clades described by Conroy and Cook (2000a). We also built a tree based on the 312-bp region of the *Cytb* gene from all ancient and modern specimens. We used this analysis to identify possible source populations of GYE voles.

Additionally, we generated a statistical parsimony haplotype network in TCS version 1.21 (Clement et al. 2000), using the 312-bp region we obtained from all ancient and modern GYE specimens ($n = 96$). We examined the distances among haplotypes and their frequencies in the modern and ancient populations. We performed analyses of molecular variance (AMOVAs) in Arlequin version 3.0 (Schneider et al. 1999) and estimated F_{ST}-values and their significance by running 1,000 permutations of haplotypes between populations. We calculated Tajima's *D* for the modern and ancient populations in order to examine patterns of nonneutral evolution.

We explored temporal genetic patterns using a variety of techniques. We used the program BEAST (Drummond and Rambaut 2003; Drummond et al. 2005) to perform a Bayesian skyline analysis that enabled us to examine population size fluctuations over the last 3,500 years based on our subfossil samples from different points in the past (''heterochronous samples''; Drummond et al. 2005). We ran BEAST 4 times independently for 200 million iterations using a separate substitution model for each codon position. This enabled us to estimate overall demographic patterns and potential fluctuations in the effective population size (N_e) that would indicate population decline or expansion.

We used an AMOVA approach to examine the distribution of genetic variation between modern and ancient populations in the GYE and among serial samples from the fossil localities. We implemented this test in Arlequin version 3.0 (Schneider et al. 1999) and estimated F_{ST}-values and their significance by running 1,000 permutations of haplotypes between populations. We used the temporal AMOVA to determine whether significant changes in genetic structure have occurred over the recent evolutionary history of the long-tailed vole. We interpreted temporal genetic structure (differences in haplotypes and their frequencies) as the signature of temporally explicit changes in population connectivity over the fossil time span. A lack of temporal genetic structure implies that the populations are genetically homogenous and similar over time.

We tested whether genetic divergences of lineages within the GYE coincided with the relatively recent time of deglaciation of the GYE (14,000 years ago). We used a conservative approach in estimation of divergence times from our data because molecular estimates of divergence times may carry extensive errors (Conroy and van Tuinen 2003; van Tuinen and Hadly 2004). A conservative approach is particularly useful for examining the correlation of haplotype divergence with the timing of Pleistocene ice ages. We set the divergence between the Southern Rockies Clade and all remaining clades in *M. longicaudus* to 0.34 ± 0.07 *SE* million years ago (mya), following Conroy and Cook (2000a). We used corrected genetic distances based on the same model previously used (Kimura 2-parameter—Conroy and Cook 2000a) and hierarchical likelihood-ratio tests to confirm that the data did not deviate significantly from a molecular clock. We then calibrated average genetic distances to estimate the average divergence time. We also estimated a minimum divergence time using the minimum (younger) value of the 95% confidence interval surrounding the calibration point. Minimum divergence estimates >0.014 mya were interpreted as rejection of the hypothesis that the diversity of voles in the GYE arose since deglaciation.

Results

Authentication of mitochondrial sequence.—We obtained complete *Cytb* sequences (1,143 bp) from 49 modern specimens and partial *Cytb* sequences (312 bp) from 35 skin and 31 subfossil specimens. Nucleotide base composition was similar to previously reported values for other *Microtus* species (26.9% cytosine, 28.35% thymine, 29.18% adenine, and 16.38% guanine [Conroy and Cook 2000b; Hadly et al. 2004]). The variation across codon positions for the modern and ancient mitochondrial sequences (3rd position: 82% of variable sites; 2nd position: 4% of variable sites; and 1st position: 14% of variable sites) was consistent with the

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expected pattern of variation in the mammalian *Cytb* gene and voles in particular (Conroy and Cook 2000a; Hadly et al. 2004). There were 78 variable sites total. Sixty-six of 78 pairwise base-pair differences were synonymous 3rd-position changes and 73 of 78 pairwise base-pair differences were transitions, consistent with the expectations of mitochondrial evolution in rodents (Irwin et al. 1991). The transition : transversion ratios of the modern and ancient data sets were 7.4 and 7.0, respectively.

Divergent sequences that appeared to be nuclear mitochondrial inserts (numts) were amplified in 6 modern specimens, using a primer pair spanning 312 bp of *Cytb*. Phylogenetic analyses revealed that these sequences (GenBank accession numbers FJ457016–FJ457021) were numts that were monophyletic and sister to the rest of our samples of *M. longicaudus*. Subsequent amplifications with different primers spanning a longer fragment (600 bp) yielded the true mitochondrial *Cytb* sequence for 3 of these samples (clustering them within the Central Clade), confirming that the shorter fragments were indeed numts. The remaining 3 samples did not yield any sequence, leading us to suspect that they were highly degraded.

Interestingly, the numt sequences had not accumulated stop codons and were not significantly different from mitochondrial GC content and substitution bias. We conclude that a single recent insertion (close to the origination of the species) into the nuclear genome likely underlies this pattern.

Phylogeography.—We constructed a maximum-likelihood tree from 111 complete *Cytb* sequences, including 71 complete sequences published by Conroy and Cook (2000a) and 40 sequences from our modern sampling in and around the GYE. The best-fit model (TVM+G+I) for this data set accounted for separate mutation rate categories for each transitional substitution type, for the proportion of invariant sites (Pinv = 0.66) and for among-site rate heterogeneity (alpha = 2.03). In agreement with Conroy and Cook (2000a), the maximum-likelihood phylogeny under this model indicated monophyly with intermediate bootstrap support for Northwest, Island, Central, and Southern Rockies clades (Fig. 2), with the Southern Rockies Clade sister to the Central and Northwest + Island clades. Most livetrapped voles from Montana and Wyoming were assigned to the Central Clade. This result is expected based on the proximity of the central Rockies to the previously described limits of this clade in southern Montana and Idaho (Fig. 1). However, voles from the Wind River Range (Wyoming) and the Beartooth Plateau (Montana) were most closely related to members of the Northwest Clade, in particular to voles from Utah and Arizona. Previously, intermediate support (56% bootstrap support) was found for placing these latter individuals in a monophyletic sister group to the Northwest + Island group, which includes individuals from Alaska, British Columbia (Canada), northern Oregon, and Washington (Conroy and Cook 2000a). Instead, we found moderate bootstrap support for placement of the Arizona, Utah, Wind River, and Beartooth individuals within the Northwest Clade, most closely related to a group of Oregon, Washington, and British Columbia voles.

We supplemented our sampling in Wyoming, Montana, Idaho, and Utah with museum specimens, owl pellet samples, and late-Holocene subfossils. Because of the older age of these specimens, we used the 312-bp fragment of the *Cytb* gene that was sequenced for all specimens. We constructed a maximum-likelihood tree using a HKY+I+G model of molecular evolution (Fig. 3). In this 2nd analysis, monophyly for the major clades was again confirmed (Northwest, Island, and Central) with the exception of the Southern Rockies Clade, which appeared as a paraphyletic group. Because none of the voles found in the GYE fell within the Southern Rockies Clade, we collapsed the latter to a single branch. For the same reason, the Island Clade and mainland Alaska voles were represented by single branches in Fig. 3. The majority of GYE and the central Rocky Mountain specimens were part of the Central Clade, including all of the Idaho samples and all but 1 of the ancient DNA samples. Museum specimens from the Black Hills extended the geographic extent of the Central Clade into South Dakota.

Concordant with the modern samples, museum specimens from Utah and Wyoming expanded the geographic range of the unique grouping of Arizona, Utah, Montana, and Wyoming voles as far east as Medicine Bow National Forest, Wyoming. We again found intermediate bootstrap support for the expanded Arizona and Utah group within the Northwest Clade (61% bootstrap support), closest to a weakly supported group of voles from British Columbia, Washington, and northern Oregon (51% bootstrap support). One unusual subfossil sample from Lamar Cave, found in a 600- to 1,200-year-old unit, constituted a unique haplotype not found in other modern or ancient samples. This specimen also fell within the Northwest Clade (61% bootstrap support) and formed a polytomy with voles from British Columbia, Alaska, Washington, and Oregon (51% bootstrap support monophyly), and the expanded Arizona and Utah Clade (63% bootstrap support monophyly). Despite the inability to place this individual among specimens of the Northwest Clade with stronger bootstrap support, our analyses revealed that it was not a member of the Central or Southern Rockies clades. Neither the grouping of individuals from the 2 fossil localities together nor from each locality alone was monophyletic. Levels of gene and haplotype diversity were similar between modern and ancient samples (see below), which were time-averaged over several thousand generations. Thus, modern, museum, and subfossil samples all reflect evidence of dispersal and genetic admixture in the central portion of the species' range.

Patterns of diversity.—We identified a total of 44 haplotypes in our samples from all locations and temporal intervals (from 96 voles). We present a haplotype network of the modern and ancient haplotypes in Fig. 4. Of the 44 haplotypes, 5 were shared by both modern and ancient populations, 12 were exclusively ancient, and 27 were exclusively modern. Twelve of the ancient haplotypes were

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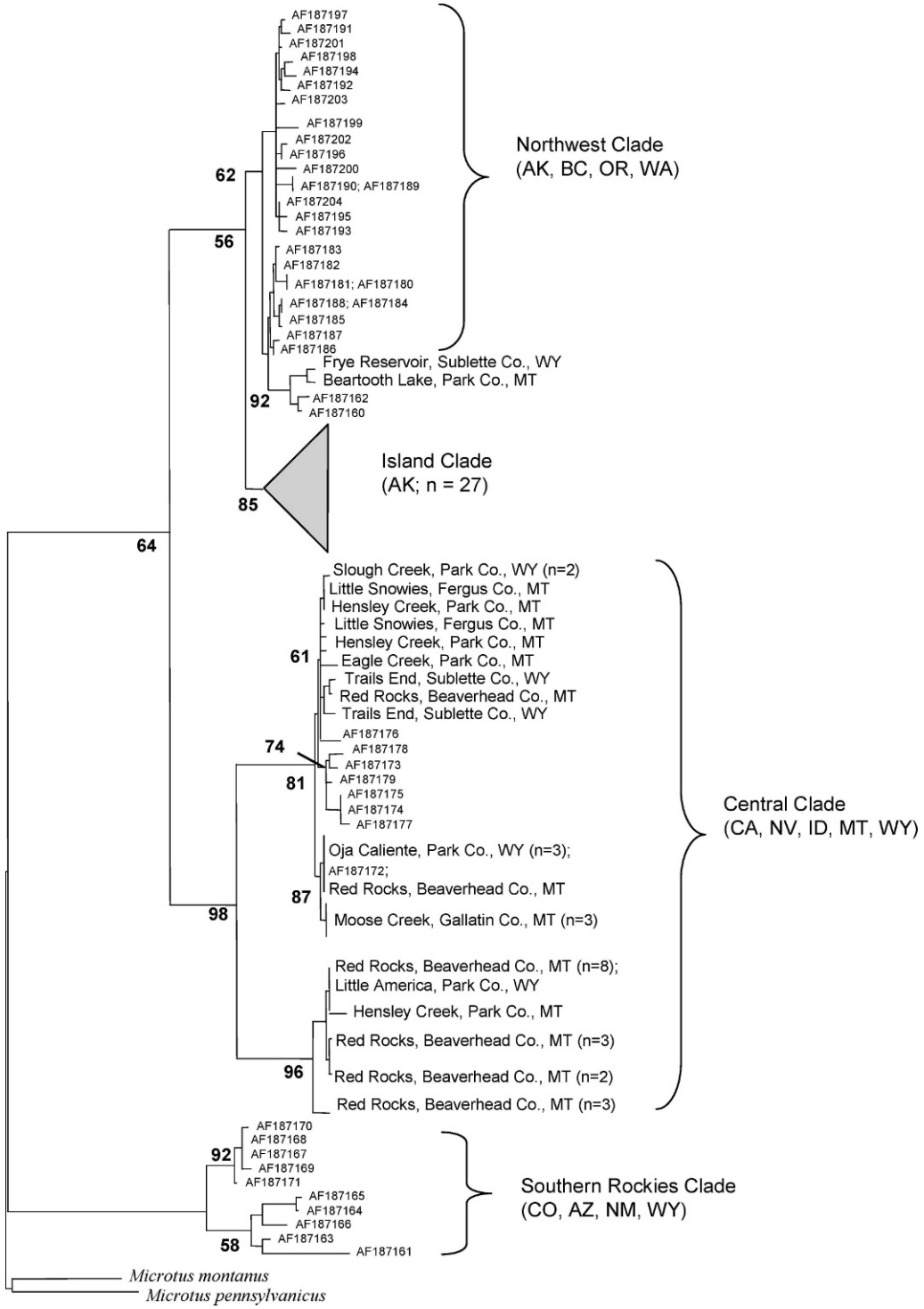


Fig. 2.—Maximum-likelihood tree of complete cytochrome-*b* data of *Microtus longicaudus* with bootstrap scores based on quartet puzzling. Previously published sequences are combined with our new samples (denoted by geographic locale).

unique, and the remaining 17 were shared with modern individuals from the central Rockies. One ancient sample was a member of the Northwest Clade (haplotype A1) and the remainder were members of the Central Clade (Fig. 4). Modern specimens were found in all 3 haplogroups. All haplotypes shared by modern and museum specimens were found within the Central Clade (Fig. 4).

Genetic diversity was high and variation was distributed uniformly among sampled groups over the last several millennia. The gene diversity seen in modern GYE voles was high (0.88) and similar to the values seen in the pooled ancient voles (0.95). The AMOVA in which we examined patterns of haplotype variation in modern GYE and ancient populations did not reveal significant population differentia-

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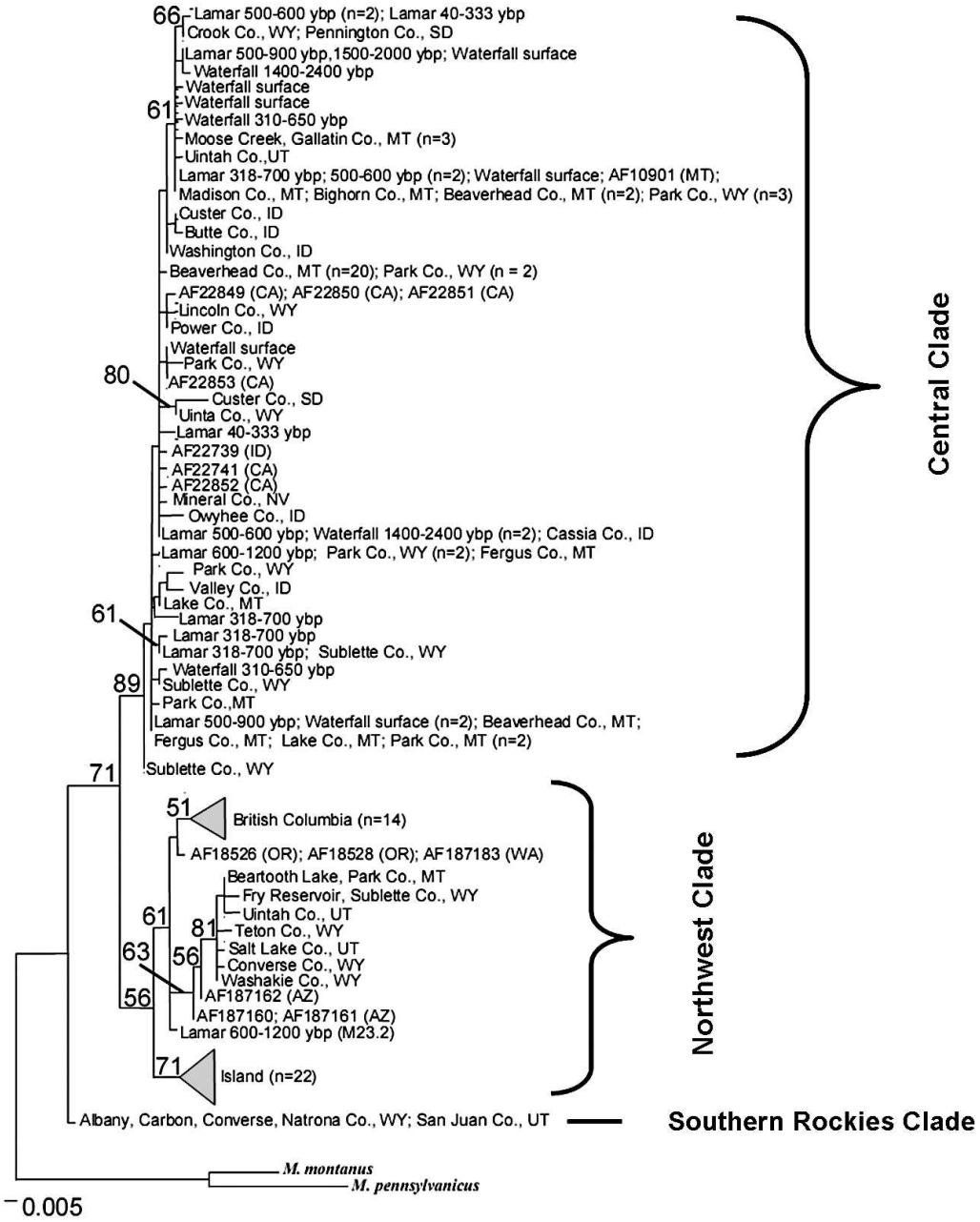


FIG. 3.—Maximum-likelihood tree based on 312 bp of cytochrome-*b* data from modern (including 52 previously published sequences from Conroy and Cook [2000a]), museum, and ancient specimens of *Microtus longicaudus*. Bootstrap scores were estimated via quartet puzzling and were similar to neighbor-joining bootstrap values (not shown).

tion through time. The modern and ancient populations had an $F_{ST} = 0.00736$ ($P = 0.26$). More than 99% of the variation in haplotype frequencies occurs within populations. Pairwise F_{ST}s between populations were very low and not significant. The calculated F_{ST} for the GYE modern–Lamar Cave comparison was 0.001 ($P = 0.477$), and the F_{ST} for the GYE modern–Waterfall Locality comparison was 0.02 ($P = 0.18$). The pairwise F_{ST} for the ancient sites was 0.009 ($P = 0.324$). When we split the ancient samples into 3 temporal populations (0–333 years ago, 310–900 years ago, and 600–2,400 years ago) we saw a similar pattern with overall $F_{ST} = 0.0086$ ($P = 0.30$). As with the modern versus ancient analysis, almost all of the genetic variation was within populations (99.14%). There were no significant pairwise F_{ST}s (data not shown).

The pooled Tajima's D for the ancient samples was -1.4 ($P = 0.09$). When the localities were examined separately, the values of Tajima's D were similar (Lamar Cave: $D = -0.10$, $P = 0.17$; Waterfall Locality $D = -0.04$, $P = 0.35$), as was the modern estimate ($D = -0.08$, $P = 0.22$). None of these values was significantly different from 0. There is no evidence of positive or purifying selection acting within our populations or of population expansion or contraction.

In our temporal analyses using BEAST, we found a stable effective population size (N_e) with a mean value approaching

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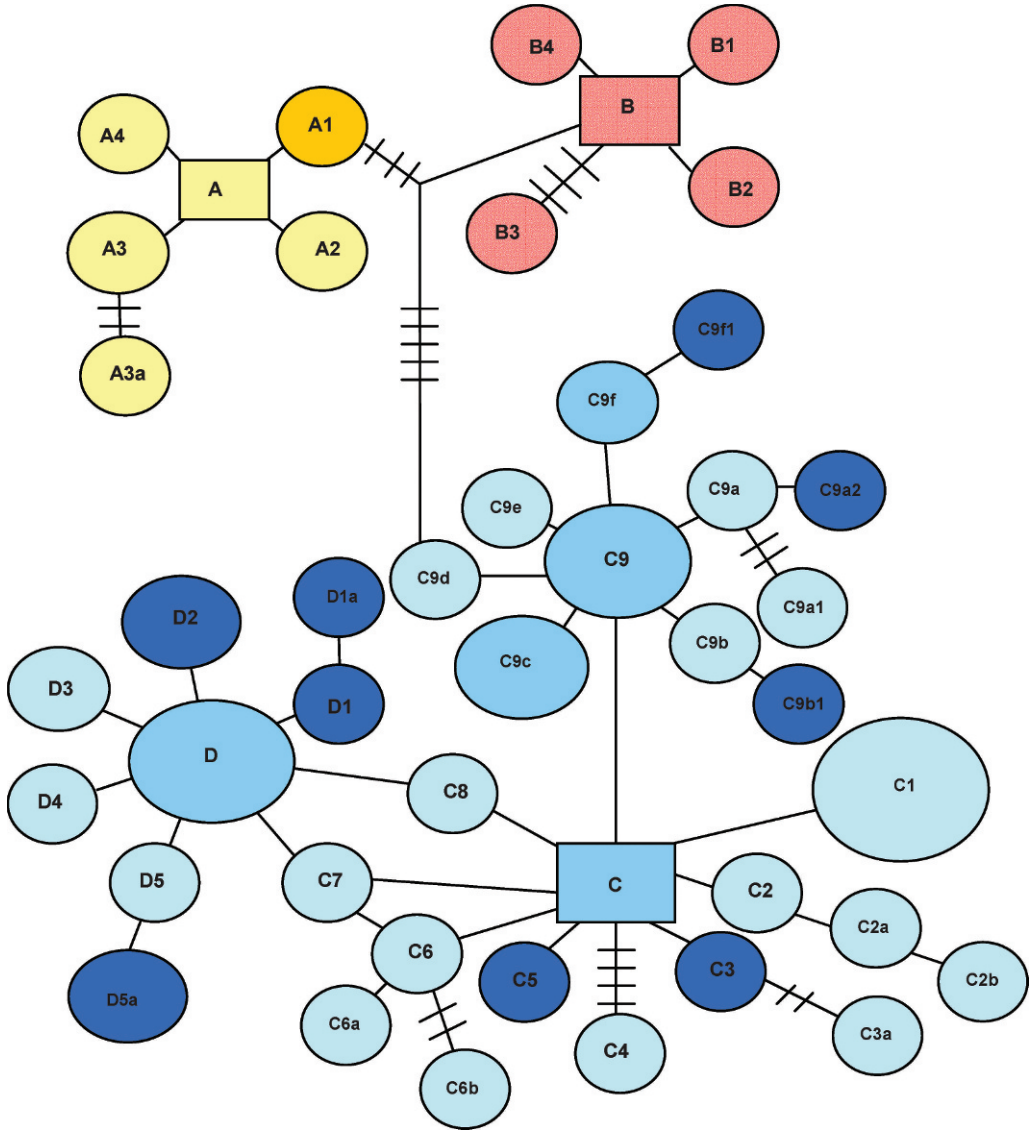


Fig. 4.—Network linking haplotypes of *Microtus longicaudus* (based on 312-bp cytochrome-*b* gene; $n = 96$). The size of the shape indicates the frequency of the haplotype. Colors indicate the major mitochondrial clade of each haplotype (blue represents individuals from the Northwest Clade; yellow represents individuals from the Central Clade; red represents individuals from the Southern Rockies Clade). Haplotypes found in the modern specimens are shown in the lightest shade, shared modern and ancient haplotypes are shown in the intermediate shade, and ancient haplotypes are shown in the darkest shade. Lines connecting haplotypes represent single base-pair differences, hatch marks across the connecting lines represent additional single nucleotide differences. Rectangles represent ''basal'' haplotypes as identified by TCS (Clement et al. 2000). The 3 basal haplotypes differ by >5% uncorrected sequence divergence.

100,000 individuals over the last 3,000 years (Fig. 5). There was no evidence of population expansion or bottlenecking in our reconstruction of the species' demographic history in this region.

Estimates of divergence time.—Based on our analysis of divergence times of the samples from the formerly glaciated GYE, we rejected the hypothesis that all genetic lineages in the GYE coalesce to the time of the last deglaciation (approximately 0.014 mya). We estimated a divergence time of 0.279 mya for the split between Central and Northwest + Island clades that are represented in our GYE samples. The minimum age of this divergence (0.088 mya) also predates the deglaciation of the GYE. We estimated the deepest divergence time within samples from the Central Clade as 0.152 mya, with a minimum divergence of 0.035 mya. The genetic diversity in the GYE samples arose before the deglaciation of this ecosystem.

Discussion

Our investigation of modern populations of long-tailed voles in the central Rocky Mountains indicates that although climatic change and geography influenced aspects of this species' range and population connectivity, the major mitochondrial lineages are not entirely spatially disjunct. The complex pattern of genetic diversity seen in the long-

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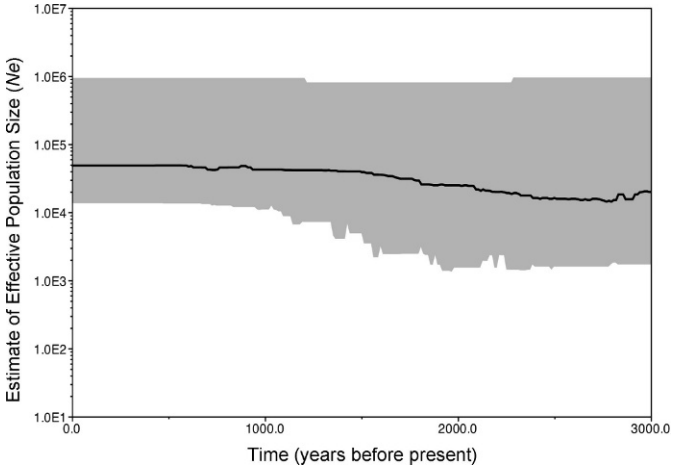


Fig. 5.—Bayesian Skyline plot showing estimated effective population size (N_e) of populations of *Microtus longicaudus* in the Greater Yellowstone Ecosystem estimated from temporal samples (Appendix II) over the last 3,000 years.

tailed vole in the central Rocky Mountains is similar to observations in locally endemic species (Brunsfeld and Sullivan 2005; Good and Sullivan 2001; Knowles 2001). However, the phylogeography of the long-tailed vole differs from the genetic patterns seen in endemic species in 1 major way: in the central Rocky Mountains, genetic diversity is high because of the presence of haplogroups that had previously been detected hundreds of miles away, in different portions of this wide-ranging species' distribution.

Examination of our GYE data reveals the co-occurrence of the Central and Northern mitochondrial haplogroups. Additional analysis of museum specimens from a 500-km-radius area surrounding the GYE suggests that the presence of individuals from the Northwest and Central haplogroups and correspondingly high levels of genetic diversity are characteristic of the larger geographic region of the central Rockies. Additionally, the Southern Rockies haplogroup occurs in this region in parts of Wyoming. Given the previous findings of Conroy and Cook (2000a), we could not have predicted this pattern.

The regional data lead us to conclude that long-tailed voles have dispersed across prominent geographic features. Individuals from the Northwest and Central clades can be found on both sides of the Continental Divide, as well as the Snake River, Green River, and Missouri River. Intensive sampling has enabled us to determine that these major rivers and mountain ranges are not complete barriers to dispersal in this species.

At the center of our study area lies the GYE. Within-population genetic diversity in the GYE is high, paralleling the high diversity in the entire region. Our late-Holocene specimens from the GYE harbor at least 2 mitochondrial lineages (Central and Northwest clades). The most common haplogroup (the Central Clade) in the ancient population also is the most common group in the region today. Furthermore, haplotypes from the Northwest Clade, previously identified in populations north of 45° latitude, were represented in Lamar Cave from 600 to 1,200 years ago and also were found in modern populations in the Beartooth Mountains, Montana, and Jackson Hole and the Wind River Mountains, Wyoming. The pattern of co-occurrence of these haplogroups in ancient and modern populations suggests stable millennial-scale coexistence of multiple mitochondrial lineages in this ecosystem.

Postglacial colonizers of the GYE are from at least 2 genetic clades with potentially different geographic origins. The phylogenetic analyses suggest that passage from the northern Rockies or the Great Plains, or both, and the Great Basin onto the previously glaciated Yellowstone Plateau was possible for this small mammal species.

In combination with the regional central Rocky Mountain data, we conclude that although dispersal from outside this region may have been possible, our data can be most parsimoniously interpreted to suggest that diverse populations containing several mitochondrial lineages were present in the vicinity of the GYE during the last glacial maximum. This would have enabled the rapid colonization of the central GYE by several haplogroups, as we have observed. Adjacent parts of the central Rocky Mountains in areas of southern Montana, Idaho, and Wyoming were not under glacial ice cover during the last glacial maximum and may have been home to genetically diverse populations of *M. longicaudus*. The lack of structure across the larger region of the central Rocky Mountains prevents us from ascertaining more about the precise source population of the postglacial colonizers. This same lack of structure illustrates a very important aspect of the species' biology: *M. longicaudus* in this area does not exhibit population-level spatial or temporal genetic differentiation.

We extend our understanding of this species' geographic distribution in this region during the last glacial maximum via analysis of spatially and temporally sampled mtDNA. We build upon the fossil data that are available to expand the probable prior distribution of the species. The distribution of fossil *M. longicaudus* since the last glacial maximum shows the species present in the southern extent of its current range, central Nevada and Utah, eastern Montana, Colorado, and Wyoming, and a few extralimital southern sites (Graham et al. 1996). The high diversity across the region in the modern and recent past (last 3,000 years) strongly suggests regional persistence of this species through the last glacial maximum.

We conclude that the central Rocky Mountains are a zone of secondary contact for long-tailed voles. The diversity of haplogroups in this region is higher than has been previously detected (Conroy and Cook 2000a). We found more support for this area as a zone of secondary contact from our molecular clock analysis. We recovered deep divergence-time estimates among our sampled populations. The haplotypes coalesced to an ancestral population that is older than the most recent glacial maximum. Therefore, we conclude that the GYE genetic diversity indicates that this area is a zone of secondary contact, although our sampling indicates that it is part of a larger area of admixture. This zone of admixture spans the central part of this species' range, which is at the biogeographic crossroads of several ecological biomes

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(Bruzgul and Hadly 2007). The high-diversity populations that have arisen since deglaciation in this region are an interesting counterexample to previously described low levels of genetic variation found in other populations occupying areas disturbed by continental ice sheets (Conroy and Cook 2000a).

The results of the Tajima's *D* and the Bayesian skyline analyses were concordant and present a picture of populations of long-tailed voles that have consistently maintained high effective population sizes temporally and spatially. In order to put the results of our temporal AMOVA into context, we performed the same analysis on previously published sequences (Hadly et al. 2004) of *M. montanus* from the same fossil localities examined in the same temporal units. In contrast to the lack of structure seen in *M. longicaudus*, significant F_{ST}-values occur in all temporal comparisons conducted on *M. montanus* (pairwise F_{ST}-values range from 0.199 to 0.245 [all *P*-values < 0.01]; $n = 53$), reflecting pulses of gene flow that occurred in this species between 1,200 and 600 years ago (Hadly et al. 2004).

High levels of genetic diversity and lack of population structure in this region indicate that the single subspecies found in this region (*M. l. longicaudus*) is not genetically distinct from other morphologically defined subspecies. The Central mitochondrial haplogroup has been found in individuals from *M. l. longicaudus*, *M. l. halli*, *M. l. abditus*, *M. l. angusticeps*, and *M. l. sierrae*; the Northwest haplogroup overlaps with the distributions of *M. l. longicaudus*, *M. l. littoralis*, *M. l. macrurus*, and *M. l. vellerosus*. A more detailed morphological analysis may provide insight into the relationship between evolutionary lineages and morphotypes in this species.

Several modern studies of genetic connectivity within the central Rocky Mountains have yielded quantitatively similar phylogeographic results. Good and Sullivan (2001) reported high levels of genetic diversity among red-tailed chipmunks in areas north of the GYE. The researchers detected a zone of secondary contact (Good and Sullivan 2001) of highly divergent mitochondrial lineages. Similarly, Knowles (2001) found that glacial and postglacial processes led to the mixing of lineages within the grasshopper *Melanoplus oregonensis* in montane regions around the GYE. These studies and our results suggest that postglacial movement in the central Rocky Mountains may foster the assembly of populations with high genetic diversity.

The large zone of admixture also resembles patterns seen elsewhere. Using ancient DNA, Hofreiter et al. (2004) observed that mobile species have dynamic phylogeographic patterns. Over intermediate timescales of hundreds to thousands of years, against a background of glacial–interglacial cycles, dispersal exerts a strong influence on population structure. This pattern has been seen in large- and small-bodied mammals in the Americas and Eurasia (Dalén et al. 2007; Hadly et al. 2004; Hofreiter et al. 2004; Leonard et al. 2000; Matheus et al. 2004; Vilà et al. 1999).

When examined at the continental scale, *M. longicaudus* appeared to display the genetic structure of a ''typical'' postglacial colonizer: genetically diverse in the south and genetically homogeneous in the north (Conroy and Cook 2000a). Our modern, museum, and subfossil data supplement these findings and reveal a geographically complex pattern in the central portion of this species' range. Collectively, these results illustrate how landscape and life-history traits result in unique patterns of postglacial recovery in different parts of a species' range.

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APPENDIX I

Specimen records of modern and museum individuals used in our analyses. Field number acronyms are: PAS: specimens collected by P. A. Spaeth; YC: specimens collected by Y. L. Chan; EH and EB: specimens collected by E. A. Hadly; MVZ: University of California Museum of Vertebrate Zoology; KU: Kansas University Natural History Museum and Biodiversity Research Center; CRCM: Washington State University Conner Museum. Field specimens were handled according to guidelines of the American Society of Mammalogists (Gannon et al. 2007) and Stanford University's Institutional Animal Care and Use Ccommittee protocol 10284. Field numbers are followed by the extraction numbers and year of collection (in parentheses). All specimens from the Greater Yellowstone Ecosystem and adjacent regions of the central Rocky Mountains were assigned a haplotype via network analysis (Fig. 4).

Modern owl pellets.—Park County, Wyoming: Lamar Valley: EB-93-204B, M 17.1 (1993), C1; EB-93-204, M 17.2 (1993), C9a1; EH-92-132, M 9.4 (1992), C3a.

Modern tissues.—Madison County, Montana: Black Butte: MVZ 202797, M 43.1 (2002), C10; Beaverhead County, Montana: Red Rock Lakes National Wildlife Refuge: PAS-03-M39, M 153.3 (2003), C1; PAS-03-BL2, M 155.4 (2003), C1; PAS-03-BL3, M 155.5 (2003), C1; PAS-03-BL8, M 155.6 (2003), C1; PAS-03-ALB, M 158.1 (2003), C1; PAS-03-AL3, M 158.2 (2003), C1; PAS-03-AL4, M 158.3 (2003), C1; PAS-03-AL6, M 158.4 (2003), C1; PAS-03-AL7, M 158.5 (2003), C9; PAS-03-AL11, M 158.6 (2003), C10; PAS-03-AL13, M 158.7 (2003), C1; PAS-04-01, M 87.1 (2004), C1; PAS-04-10, M 87.2 (2004), C1; PAS-04-11, M 87.3 (2004), C1; PAS-04-21, M 87.6 (2004), C10; PAS-04-27, M 88.1 (2004), C1; PAS-04-44, M 88.10 (2004), C1; PAS-04-48, M 88.11 (2004), C1; PAS-04-29, M 88.2 (2004), C1; PAS-04-33, M 88.4 (2004), C1; PAS-04-35, M 88.5 (2004), C1; PAS-04-36, M 88.6 (2004), C1; PAS-04-37, M 88.7 (2004), C1. Fergus County, Montana: Little Snowy Mountains: YC-03-064, M 71.2 (2003), C9c. Gallatin County, Montana: Moose Creek: YC-04-007, M 61.7 (2004), C8; YC-04-008, M 61.8 (2004), C8; YC-04-009, M 61.9 (2004), C8. Lake County, Montana: Swan Valley: EH-02-07, M 38.2 (2002), C9; EH-02-08, M 38.3 (2002), C9a. Park County, Montana: Beartooth Lake: YC-03-095, M 56.6 (2003), A3; Eagle Creek: YC-03-103, M 51.2 (2003), C9e; Hensley Creek: YC-03-044, M 57.1 (2003), C9; YC-03-056, M 57.4 (2003), C9; YC-03-084, M 71.3 (2003), C9; YC-03-068, M 79.10 (2003), C9. Park County, Wyoming: Little America: YC-04-078, M 74.2 (2004), C1; Ojo Caliente: YC-04-029, M 70.5 (2004), C10; YC-04-030, M 71.4 (2004), C10; YC-04-032, M 71.5 (2004), C10; Slough Creek: YC-04-127, M 71.8 (2004), C9c; YC-04-022, M 70.2 (2004), C9c. Sublette County, Wyoming: YC-04-185, M 72.8 (2004), A3a; Big Sandy: YC-04-189, M 76.6 (2004), C9f; Fry Reservoir: MVZ 202803, M 44.4 (2002), C9d; Trail's End: YC-04-212, M 77.4 (2004), C9f.

Museum skins.—British Columbia, Canada: Atlin: MVZ 34451, M 66.3 (1924); MVZ 34452, M 66.4 (1924); Desolation Sound: MVZ 129359, M 68.3 (1960); MVZ 129360, M 68.4 (1960); Kingcome Inlet: MVZ 129362, M 68.5 (1960); Stikine River: MVZ 30810, M 67.1 (1919); MVZ 30812, M 67.3 (1919). Butte County, Idaho: Oakley: CRCM-041330, M 82.8 (1949), C6. Cassia County, Idaho: Mt. Harrison: CRCM-041329, M 82.7 (1949), C. Owyhee County, Idaho: Silver City: CRCM-041310, M 82.5 (1950), C4. Power County, Idaho: Heglar Pass: CRCM-041345, M 82.4 (1949), C2. Valley County, Idaho: Little Payette Lake: CRCM-041307, M 82.3 (1949), C6b. Washington County, Idaho: Brownlee R.S.: CRCM-041304, M 82.2 (1954), C7. Big Horn County, Montana: Custer: KU 18407, M 90.3 (1946), C10. Custer County, South Dakota: Bear Creek: CRCM-041318, M 82.6 (1949), C6a; Custer: KU 87871, M 90.4 (1961). Pennington County, South Dakota: Hill City: KU 101484, M 90.5 (1965). Salt Lake County, Utah: Solitude Ski Resort: CRCM-04608, M 82.1 (1967). San Juan County, Utah: Monticello: KU 63312, M 90.1 (1954); KU 63313, M 90.2 (1954). Uintah County, Utah: Vernal: KU 38082, M 91.1 (1950); KU 38083, M 91.2 (1950). Albany County, Wyoming: Brown's Peak: KU 17393, M 91.3 (1946),

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B3; Class Lake: KU 91251, M 91.4 (1947), B1. Carbon County, Wyoming: Rawlins: KU 20632, M 92.1 (1947), B. Converse County, Wyoming: Glenrock: KU 81805, M 92.3 (1959), A4; Orin: KU 42124, M 92.2 (1951), B4. Crook County, Wyoming: Moorcroft: KU 87873, M 92.4 (1961), C10e. Lincoln County, Wyoming: Alpine: KU 37807, M 92.6 (1950), C2a. Natrona County, Wyoming: Casper: KU 42149, M 92.7 (1951), B2; KU 42150, M 92.8 (1951), B. Sweetwater County, Wyoming: Rock Springs: KU 42153, M 93.3 (1951), A3. Teton County, Wyoming: MVZ 136903, M 20.4 (1928), A2. Uinta County, Wyoming: Robertson: KU 26272, M 93.4 (1948), C2b. Washakie County, Wyoming: Tensleep: KU 20666, M 93.5 (1947), A.

Appendix II

Subfossil specimens used in analyses. For each site, all specimens are listed by their extraction number. Age (years ago) appears in parentheses, followed by the haplotype designated via network analysis (Fig. 4).

Waterfall Locality.—M 30.3 (0), C9; M 30.4 (0), C10b; M 31.1 (0), C3; M 31.2 (0), C10; M 31.3 (0), C10d; M 32.1 (0), C10c; M 32.2 (0), C9; M 32.4 (310–650), C10a; M 3.3 (310–650), C9b1; M 32.3 (1,400–2,400), C10a1; M 3.1 (1,400–2,400), C; M 3.2 (>2,400), C10b.

Lamar Cave.—M 29.2 (40–333), C10e1; M 29.3 (40–333), C5; M 37.1 (318–700), C10; M 24.2 (318–700), C9a2; M 25.1 (318–700), C9f1; M 34.3 (318–700), C9f; M 24.3 (500–600), C10; M 25.3 (500–600), C10e1; M 35.1 (500–600), C; M 35.5 (500–600), C10e1; M 16.2 (500–600), C10; M 13.1 (500–900), C9; M 13.2 (500–900), C10; M 8.1 (500–900), C10b; M 23.1 (600–1,200), C9c; M 23.2 (600–1,200), A1; M 5.2 (1,500–2,000), C10b.

Appendix III

Summary of data from cloning reactions performed on ancient sample M 23.2. Data presented are 20 clones of the fragment (Cleth 37L/Arvic 08L). Six different sequences were recovered: 8 clones (40%) matched the original amplification; clone 20 differed at 2 positions (nonsynonymous transitions); 3 different haplotypes differed at 3 positions (clones 9–12: 2 nonsynonymous transitions, 1 synonymous transition; clones 18–19: 3 nonsynonymous transitions; clones 14–17: 1 nonsynonymous transition, 2 synonymous transitions); clone 13 differed at 4 positions (3 nonsynonymous transitions, 1 synonymous transition).

								1	1	1
		2	2	4	7	8	8	0	0	1
	2	4	7	5	6	0	7	0	4	4
M 23.2 consensus	C	G	G	A	C	C	G	C	C	G
Clone 1	.	.	.	.	.	.	.	.	.	.
Clone 2	.	.	.	.	.	.	.	.	.	.
Clone 3	.	.	.	.	.	.	.	.	.	.
Clone 4	.	.	.	.	.	.	.	.	.	.
Clone 5	.	.	.	.	.	.	.	.	.	.
Clone 6	.	.	.	.	.	.	.	.	.	.
Clone 7	.	.	.	.	.	.	.	.	.	.
Clone 8	.	.	.	.	.	.	.	.	.	.
Clone 9	.	.	.	.	T	T	.	T	.	.
Clone 10	.	.	.	.	T	T	.	T	.	.
Clone 11	.	.	.	.	T	T	.	T	.	.
Clone 12	.	.	.	.	T	T	.	T	.	.
Clone 13	.	.	.	.	T	T	A	T	.	.
Clone 14	T	.	.	G	.	.	.	.	T	.
Clone 15	T	.	.	G	.	.	.	.	T	.
Clone 16	T	.	.	G	.	.	.	.	T	.
Clone 17	T	.	.	G	.	.	.	.	T	.
Clone 18	.	A	A	.	.	.	.	.	.	A
Clone 19	.	A	A	.	.	.	.	.	.	A
Clone 20	.	A	.	.	.	.	.	.	.	A

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