DISCRIMINATION BETWEEN *MICROTUS LONGICAUDUS* AND *MICROTUS MONTANUS* USING CRANIAL MORPHOLOGY, EXTERNAL MEASUREMENTS, DNA SEQUENCING, AND DISCRIMINANT FUNCTION ANALYSIS

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Abstract

Montane and Long-tailed voles (*Microtus montanus* and *Microtus longicaudus*) are notoriously difficult to distinguish through external characteristics and measurements in the field, particularly among juveniles. Being able to accurate identify these species is important, due to their central role in ecosystems (as keystone species) and as indicator species of climate change (due to their high sensitivity to climate change). The present study assessed three methods for distinguishing the two species: cranial (skull-based) and external measurements, the presence of additional skull characteristics, and DNA sequencing through examining 372 specimens in the University of Colorado Natural History Museum collection and 52 specimen from recent collection by the McCain lab (2010-2011). Three external measurements (including total specimen length, tail length, and hind foot length), nine cranial measurements, and four skull characteristics were employed. A subset of the sample population (*M. longicaudus*, n = 5; *M. montanus*, n = 17) was verified with genetic methods. Through assessing combinations of external and cranial measurements using discriminant function analysis, I determined which would best identify species regardless of age. Models with only external measurements left many juveniles misclassified (4.6% misclassified), models with only cranial measurements were more accurate (0.5% misclassified), and models with all twelve characters were most accurate (0% misclassified). All specimens were correctly identified using a best-fit model of three measurements (tail length and the breadth of two specific skull bone formations; 0% misclassified). This model correctly classified the remaining 28 juvenile specimens of unknown identity with an average fit of 99.8%.

Introduction

Montane and Long-tailed voles (*Microtus montanus* and *Microtus longicaudus*) are notoriously difficult to distinguish through external characteristics and measurements in the field, particularly among juveniles (Hall, 1981; Hoffmeister, 1986; Smolen and Keller, 1987; Sera and Early 2003; Armstrong, 2011). Being able to accurately identify these two species is important, not only to provide a sound basis for scientific study of both of these species, but also due to their central role in ecosystems and their high sensitivity to climate change (Moritz et al, 2008; McGuire 2011).

The goal of the present study was to determine which measurements (external or cranial) and which skull characters, would most accurately differentiate between *M. longicaudus* and *M. montanus*, particularly among small or juvenile specimens. My purpose was to develop a method to distinguish all *M. longicaudus* and *M. montanus* individuals, regardless of size (juvenile or adult), using either univariate or multivariate statistics from cranial and external characters and verifying a subset of juvenile specimen identifications through genetic data from select research specimens. Thus, the main questions addressed herein included:

(1) Is it possible to robustly identify *M. longicaudus* and *M. montanus* regardless of size, with only (a) external measurements, (b) skull measurements, or (c) skull characters?

(2) Is it possible to robustly identify *M. longicaudus* and *M. montanus*, regardless of size, with a multivariate model using both external and skull measurements?

(3) Can the models in (1) and (2) be used to robustly identify juvenile specimens that appear to be either *M. longicaudus* or *M. montanus* or is DNA analysis necessary for robust identifications?

Background

The Montane vole (*Microtus montanus*) and the Long-tailed vole (*M. longicaudus*) occur sympatrically, occupying similar habitats within their geographic and elevation ranges (Fig. 1, Hall 1981; Hoffmeister 1986). Where *M. longicaudus* and *M. montanus* occur sympatrically, individuals are difficult to differentiate because of similarity in external, dental, and cranial (skull) morphologies, and sexual dimorphism (different physical characteristic of the sexes) is slight (e.g., Sera and Early 2003). Historically, tail length compared to body length has been a widely used external indicator of species differentiation between the *M. longicaudus* (Long-tailed vole) and *M. montanus* (Montane vole). In adult *M. longicaudus*, the tail is greater than one-third the total length of the animal or more than 40% the length of the head and body (Hoffmeister 1986; Smolen and Keller 1987). In contrast, in adult *M. montanus* the tail is less than 50% of body, usually 29−39% of the head and body, or about twice the size of the hind foot (Hoffmeister 1986; Zeveloff and Collett 1988; Foresman 2001). If identification uncertainty remained, skull measurements (Table 1) and skull characters were examined, including the visibility of incisors when viewing the skull from above, constriction of the incisive foramina, and presence of a temporal groove (Fig. 2, 3; Hall et al. 1981; Hoffmeister 1986; Smolen and Keller 1987; Sara and Early 2003).

Mammalian systematics, the study of the evolutionary history and classifications of the mammals, traditionally uses only adult specimens in creating parameters for identification in (Wilkins, 2009). The standards and models used to identify *Microtus longicaudus* and *M. montanus* were developed by Hall (1981), Hoffmeister (1986), and Armstrong (2011) through measuring characters of adult specimens. Because the cranial measurements and characters, and external measurements of adult specimens are fully formed, this has traditionally led to confident

and correct identification between *M. longicaudus* and *M. montanus*. However, small specimens (juveniles) can be indistinguishable in many of these characteristics, which can lead to misidentification (Fig. 4a). Even among adults of these two species, character measurements overlap and no single measurement can be used with absolute confidence to identify individuals of the two species where they occur sympatrically (e.g. Hoffmeister 1986; Sara and Early 2003; Table 1). Age classes, usually identified by molar eruption and wear in rodents, are not easily distinguishable because the molars of most voles are continuously growing (Anderson 1959; Sara and Early 2003). Therefore, in voles, overall body size (total length or weight) is used to infer age (e.g., juvenile, adult).

Several other methods for species identification exist for species with strongly overlapping external morphology. Analysis of skull measurements using multivariate statistical techniques designed specifically for maximal separation between closely related groups has been conducted for species of harvest mice, *Reithrodontomys* (e.g., Hoffer et al. 1999), but these discriminate function techniques have not previously been used in studies of Montane or Long-tailed voles. McGuire (2011) used geometric morphometric analysis to directly compare molar shape differences between *M. montanus* and *M. longicaudus* (Kendall 1977). This method of identifying *M. longicaudus* and *M. montanus* using two-dimensional images of a specimen's molars is particularly useful when dealing with partial skulls found in the fossil record and skull remains found in owl feces (McGuire 2011). Stangl et al. (1993, 2004) have used similar cranial morphometrics methods to identify individuals of *Reithrodontomys meglotis* and *R. montanus*, as well as *Microtus ochrogaster*. Although the latter methods are highly accurate, they have not yet become standard practice because they require specialized imaging equipment and software and are time intensive, therefore making them prohibitive methods for many researchers. In other

attempts to reliably identify rodent species, Stangl et al. (1993) attempted identifications between two *Reithrodontomys* species using a single cranial measurement, interorbital breadth. Although useful in identifying some problematic specimens, the latter parameter was not successful in identifying all specimens. For *M. longicaudus* and *M. montanus*, Hoffmeister (1986) compared several cranial measurements of specimens in Arizona using univariate statistics for species identification. According to standard systematics practices, he constrained his analyses to adult rodent specimens of similar sizes and was able to identify most individuals, but did not make any attempt to account for geographic or sub-adult variation. DNA analysis is always a robust way to identify specimens, but remains restrictive due to time and cost. It would be possible to identify specimen based on karyotypes (size, shape, and number of chromosomes) if fresh specimens were available (*M. longicaudus*: 2n = 64, Lemskaya et al. 2010; Frey 2009; *M. montanus*: 2n=24; Sera and Early 2003). However, in the present study this was not possible because I relied on previously captured museum specimens. Because research in the field and lab needs an efficient and robust manner to identify juveniles of the species, a more effective method of identifying *M. longicaudus* and *M. montanus* using external and cranial measurements is a necessity and was developed in the present thesis.

Material and Methods

Specimens

I examined all available specimens of *Microtus longicaudus* and *Microtus montanus* in the Museum of Natural History (UCM) at University of Colorado at Boulder and from recent collecting efforts (lab of Dr. Christy McCain). This included 372 UCM specimens and 52 McCain specimens for 424 specimens in total. We used specimens of all age classes and removed specimens with incomplete data due to broken skulls or missing external

measurements. The resulting dataset included 95 *M. longicaudus* (14 McCain specimens, 81 UCM specimens) and 139 *M. montanus* (35 McCain specimens, 104 UCM specimens), and 28 unidentified, juvenile specimens that could be either species (McCain specimens).

The UCM skulls are all from Colorado and were collected within the last 125 years. Specimens from recent collecting efforts in the McCain lab (2010−2012) were from the San Juan Mountains and Front Range along four elevational transects (Fig. 5). Experts had previously made the identifications of all specimens from the UCM. Any skulls that were broken and thus were missing any standard cranial measurements were excluded from multivariate models. Twenty-four difficult to identify juvenile McCain specimens were selected for DNA analysis.

All specimens were divided into three groups for analyses: (1) expert-identified UCM specimens specimen used for developing the multivariate model (n=185), plus a subset of the DNA analyzed specimens (n=20), (2) difficult to identify juvenile McCain specimens for DNA analysis (n=24), and (3) difficult to identify, hypothesized juvenile McCain specimens for identification using the best discriminant model (n=29).

Cranial Measurements

Nine skull measurements were taken for each *Microtus* skull using electronic calipers (*SPI Digital Caliper 14-792*) to the nearest 0.01 mm. A subset of skulls were measured multiple times until all skull measurement values were consistent, and thereafter each skull was measured twice to verify accuracy. Standardized measurements (Fig. 6) for *Microtus* followed Hoffmeister (1986) and Conroy and Gupta (2001). The measurements include condylobasal length (CBL) = length between occipital condyles to anterior tip of nasals; condylobasilar length (CNL) = length between occipital condyles to posterior tip of front left incisors; occipital nasal length (ONL) = length between occipital condyles to anterior tip of nasals; nasal length (NAL) = greatest length

of nasal bone (from nasal tip to suture); zygomatic breadth (ZYB) = greatest breadth of skull at zygomatic arches; mastoid width (MAW) = width of skull at the lambdoidal ridge; prelamboidal breadth (PLB) = width of skull at the prelambdoidal ridge; interorbital constriction (IOB) = narrowest part of the constriction; upper molar alveolus (MAL) = greatest length of the upper, left molar alveolus. The original collector(s) had gathered external measurements of tail length, total length and hind foot length (mm; Table 1) for both UCM and McCain lab specimens. Weight (g) and ear length (mm) were missing for many of the UCM specimens, and the latter specimens were thus excluded in analyses and models.

Skull Characters

Presence or absence of four skull characters suggested to be able to differentiate between *M. longicaudus* and *M. montanus* were examined (Hoffmeister, 1986; Hall, 1981). These include (Fig. 2; Fig. 3) the constriction of incisive foramen, presence of a temporal groove, visibility of incisors as seen from directly above the skull, and distance between nasal and pre-maxillary sutures (Hoffmeister, 1989; Naughton, 2012). Presence of a constricted incisive foramen, an obvious temporal groove, visibility of incisors, and comparison of maxillary to pre-maxillary sutures have previously been found to indicate *M. montanus* (Hall, 1981; Hoffmeister, 1986; Armstrong, 2011). To determine statistical significance, a non-parametric Fisher's exact test was used for univariate analyses of skull characteristics due to small sample sizes of count data (Table 2).

DNA Extraction and Molecular Protocols

For unidentified, juvenile specimens, DNA was extracted from neck muscle tissue preserved in ethanol following standard phenol/chloroform procedure (Sambrook *et al*., 1989). DNA was extracted using the *DNeasyTM Purification of Total DNA from Animal Tissues Spin-*

Column Protocol (QIAGEN Inc., Venlo, Netherlands), with the modification of decreasing the volume of buffer AE to 150 µL in step 7 to increase DNA concentration. DNA Primers L 14841 (forward) and H 15149 (reverse) were used to amplify of the mitochondrial gene Cytochrome Oxidase II (Kocher et al., 1989). All specimens were compared using the cytochrome oxidase II gene (Avise et al. 1987; Liu and Beckenbach 1992). Polymerase chain reaction (PCR) was completed using 1µL DNA, 8µL 1X Master Mix (5 Prime), 1 µL of each Primer, and 10µL nuclease free water. Each PCR cycle consisted of 93°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes, and repeated 35 times. For more detail, see texts (e.g. Maekawa et al., 1999; Jones et al., 2010) on standard methods for extracting and sequencing DNA. Crude PCR samples were cleaned and sequenced by Functional Biosciences, Inc. (Madison, WI). The returned sequences were then visually edited for sequencing errors using the program Sequencher, version 4.6 (Gene Codes Corporation, Ann Arbor, Michigan) and DNA trees were constructed in FigTree v1.3.1 (Appendix 1; Tree Figure Drawing Tool, Version 1.3.1 2006-2009, Andrew Rambaut Institute of Evolutionary Biology, University of Edinburgh).

Statistical Analysis

JMP® Pro 10.0.1 (SAS Institute Inc. 2012) was used for all statistical analyses, except for the Fisher's exact test that was run using QuickCalcs (GraphPad Software, Inc. 2013). A discriminant function analysis with stepwise variable selection was employed to assess the best sets of variables to distinguish between *Microtus longicaudus* and *M. montanus*. I first ran an analysis on specimens from the UCM, including those I had verified via DNA sequencing (n=205), using all twelve measurements of the characters of the specimens. I then repeated this procedure with just the three external characters of (1) total length, (2) tail length and (3) hind foot length, and then again with only nine cranial characters of (1) condylobasal length, (2)

condylobasilar length, (3) occipital nasal length, (4) nasal length, (5) zygomatic breadth, (6) mastoid width, (7) prelamboidal breadth, (8) interorbital constriction, and (9) upper molar alveolus. Finally, to find the best model with the fewest variables, I ran a step-wise discriminant function analysis on all twelve measurements and, stepping forward and back, determined the critical external and cranial measurements needed to identify all specimens accurately. Using the information gathered from discriminant scores of *Number Misclassified*, *Percent Misclassified*, and the *-2LogLikelihood* (Table 3), I then applied the best-fit model to the unidentified skulls from the McCain collection to confirm their species identification. Better discriminant function models have the fewest *Percent Misclassified*, and the lowest *-2LogLikelihood* values. In using the model for classification of unidentified specimens, those individuals with significant fits to one of the species are assessed by probability of correct classification (e.g., strong identification values > 70%).

Results

Measurements of both external measurements and skull characters show substantial overlap between *Microtus longicaudus* and *M. montanus* (Table 1, Fig. 4), while skull measurements also substantially overlapped, particularly for skulls of small individuals, prelamboidal measurements exhibited the least overlap between *M. longicaudus* and *M. montanus* (Table 1).

The four skull characters traditionally suggested for differentiating between *M. longicaudus* and *M. montanus* did not show clear delineation among all specimens (Fig. 2 and Fig. 3). For the incisive foramen (Fig. 3), *M. montanus* should show constriction, whereas *M. longicaudus* should not. Of 141 *M. montanus* skulls examined, 73% showed constriction, 20% showed no constriction, and 7% were indistinguishable. Of 100 *M. longicaudus* skulls

examined, 12% showed constriction, 79% showed no constriction, and 9% were indistinguishable. With regard to visibility of incisors looking straight down on the skull (dorsal view), *M. montanus* should have visible incisors, whereas *M. longicaudus* should not. In *M. montanus,* 95% of the incisors were clearly visible and 5% were not. In *M. longicaudus*, 20% of the incisors were clearly visible and 80% were not. The presence of a temporal groove indicates *M. montanus* and absence indicates *M. longicaudus*. Among *M. montanus* skulls, 82% had a temporal groove, 16% did not, and 2% were indistinguishable. Among *M. longicaudus* skulls, 6% had a temporal groove, 92% did not, and 2% were indistinguishable (Table 2). There was little variation among specimens using pre-maxillary to nasal sutures length comparisons, and this character thus proved to be inconclusive and was not further analyzed. All three skull characters examined were significantly different between *M. longicaudus* and *M. montanus* (Fisher's exact tests: all p-values < 0.0001; Table 2). Regardless of statistical significance, many skulls were not diagnosable to species level using these three characters because of disagreement among characters in taxon identification or because a character state was indistinguishable.

Using DNA, 17 juvenile, unidentified specimens were identified as *M. montanus*, while six were identified as *M. longicaudus.* One specimen was identified as *Microtus mexicanus* and was excluded from models (Appendix 1). Measurements of DNA-identified specimens of *M. longicaudus* and *M. montanus* were incorporated into multivariate models.

Comparing cranial measurements and external measurements between *M. longicaudus* and *M. montanus* through univariate statistics was largely inconclusive. The best comparison of external measurements, tail-length to total length, showed deep overlap in measurements between species of small specimens (Fig. 4). In cranial (skull) measurements, the greatest differentiation between species was found comparing prelamboidal breadth to condylobasal

length, which still contained much overlap (Fig. 8). The best univariate model was found through comparing both external and cranial measurements of prelamboidal and condylobasal length (Fig. 9). However, even this best-fit univariate model showed overlap between the two species and further multivariate analysis was needed.

The step-wise discriminant function model including only external measurements (total body length, tail length, and hind foot length) to differentiate *M. longicaudus* and *M. montanus* specimens was the least accurate (4.569% misclassified, -2LogLikelihood of 43.84). The discriminant function model including only the nine skull measurements was slightly more accurate (0.488% misclassified, -2Logliklihood of 8.343), but still misidentified some specimens. Using all twelve measurements (external and skull) gave the most accurate model with the lowest -2LogLikelihood (0% misclassified, -2Logliklihood of 0.14). However, using the stepping forwards and backwards function of variable selection in JMP discriminant function stepwise models, I found that only three measurements (tail length, mastoidal breadth, and prelamboidal breadth) are necessary to accurately identify specimens (Table 3, Fig. 7). Using the most accurate model (all measurements), and all remaining unidentified juveniles (n = 28) were all correctly identified to species with an average probability of 99.9%. Similarly, using the best-fit model (tail length, mastoidal breath, and prelamboidal breath), all remaining unidentified juveniles were also correctly identified to species with an average probability of 99.8%.

Discussion

External characteristics, such as fur color or overall size, do not clearly identify most individuals of *Microtus longicaudus* or *Microtus montanus* where they occur sympatrically (Hall 1981; Hoffmeister 1986). The traditional indicator, tail length, works well among most adults

(Hall, 1981; Hoffmeister, 1986; Smolen and Keller, 1987; Sera and Early 2003; Armstrong, 2011). However, particularly in juveniles or individuals with tail injuries, this characteristic is less than definitive, rendering some identifications questionable. Previous to the present study, specimens such as these could not be identified with confidence without DNA or karyotypic data because of the ambiguity of their external and skull measurements (Howell 1924; Hoofer et al. 1999; Fig. 4, 8, and 9).

Cranial measurements alone cannot be used to correctly identify all individuals of *M. longicaudus* and *M. montanus* in Colorado because all measurements overlap substantially (Hoofer et al. Stangl et al. 1993; Table 1). For larger adults, tail length and prelamboidal breath could potentially be used due to lower overlap in values between the two species (Smolen and Keller 1987, Sera and Early 2003). Since univariate analysis of external and cranial measurements show overlap between species across ages and sizes, and particularly among juveniles, no single measurement can be used with confidence to identify individuals, even among adult specimens (Fig. 4, 8, and 9).

Skull characters (e.g., presence of a temporal groove, visibility of incisors, and constriction of the incisive foreman) have historically been used as reliable indicators between *M. longicaudus* and *M. montanus* (Hall 1981; Hoffmeister 1986; Armstrong 2011). However, these characters were unreliable even among the skulls from the UCM used in multivariate models. Among the reliably detected characters, the visibility of incisors proved to be the clearest of the characteristics examined, but were not definitively conclusive in species identification for all specimens. Skull characters were too subjective to be useful in robust species identification, particularly in juveniles, whose skulls are still forming.

Regardless of size, discriminant function models computed for either the twelve-character or three-character models facilitate identification of all specimens of the two species from the San Juans and Front Range of Colorado. Either set of discriminant multipliers provides a dependable method to identify individuals *M. longicaudus* and *M. montanus*. As found in Hoofer et al.'s (1999) work with *Reithrodontomys*, having two models of different numbers of variables allows flexibility for specimens that may be missing some data fields due to a broken skull or a missing external measurement.

When the twelve-character model was applied to the remaining unidentified, juvenile specimens, all specimens were conclusively assigned to a species with the model. The three-character (best-fit) model was equally at robust in identifying unknown specimens as the full twelve-variable model. Comparison of discriminant scores, using both the twelve-character and the three-character models of the specimens of the two species from areas other than Colorado would serve to underscore the effectiveness of this discriminant analysis in distinguishing between *M. longicaudus* and *M. montanus* across their geographic ranges.

To resolve problematic identifications between these two sympatric species, independent researchers can measure a combination of cranial characters and incorporate the values into the model developed in this study for identification. If measurements are missing or unavailable to use in this model, future researchers can develop their own model through similar step-wise multivariate statistics (Hoofer et al. 1999). It is important to note that ideally the same researcher should measure all the characters for the sake of consistency in developing a model. Due to variability of measurement techniques, this is important in order to determine the proper measure of relationships between these measurements and for the model to produce accurate results.

Conclusions

The use of discriminant functional analysis holds enormous potential for identifying *Microtus longicaudus* and *M. montanus,* particularly among juveniles. Although other methods, such as geometric morphometrics, karyotyping and DNA analyses are also employable, field scientists studying *Microtus* will continue to use efficient and cheap measurement techniques that are employed by museums worldwide. Therefore, better analysis of mensural data is needed to confidently identify species. DNA analysis does provide conclusive results, but is presently costly and time consuming. To better understand overall mammalian ecology and how changing climate, human interaction, and time has affected mammals, accurate species identification is critical. It is necessary to distinguish with certainty between *Microtus longicaudus* and *M. montanus* to maintain accurate identification of these two sympatrically occurring species for future scientific research, and to help researchers develop accurate models for identifying juveniles of other mammalian species. In the larger picture, rodents of the genus *Microtus* can provide essential information about how small mammals respond to historic environmental changes, because they are abundant now, well represented in the Quaternary fossil record, and known to have experienced range shifts in response to recent and past environmental changes (McGuire 2011).

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References

Anderson, S. (1954). Subspeciation in the meadow mouse, *Microtus montanus*, in Wyoming and Colorado. University of Kansas Publications. *Museum of Natural History.* 7, 489–506.

Anderson, S. (1959). Distribution, variation, and relationships of the montane vole, *Microtus montanus*. University of Kansas Publications. *Museum of Natural History.* 9, 415–511.

Armstrong, D. M., J. P. Fitzgerald, and C. A. Meaney. 2011. *Mammals of Colorado.* 2nd edition, Denver, CO.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N.C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review Ecolology Systems*. 18, 489–522.

Bailey, V. (1900). Revision of American voles and the genus Microtus (No. 17). *Government Print Off*.

Colorado State University. (2003). "Managing voles in Colorado" (On-line). *Colorado State University Cooperative Extension.* 4/11/03. http://www.ext.colostate.edu/PUBS/NATRES/06507.html.

Conroy, C. J., & Cook, J. A. (2001). Phylogeography of a post‐glacial colonizer: *Microtus longicaudus* (*Rodentia: Muridae*). *Molecular Ecology*. 9(2), 165-175.

Cosens, L. (2004). "Microtus longicaudus" (On-line), Animal Diversity Web. Accessed March 19, 2013 at http://animaldiversity.ummz.umich.edu/accounts/Microtus_longicaudus/

Ergon, T, X. Lambin, & N.C. Stenseth. (2001). Life-history traits of voles in a fluctuating population respond to the immediate environment. *Nature.* 411, 1043-1045.

Foresman, K. R. 2001. The wild mammals of montana. *American Society of Mammalogists*. Special Publication No. 12: Lawrence, KS, 278.

Frey, J.K., B.J. Frey, and D.W. Moore. (2009). Karyotypes of the long-tailed vole (*Microtus longicaudus*) in isolated mountain ranges of the American southwest. *Western North American Naturalist.* 69(3), 388-390.

Grinnell, J., J. S. Dixon, and J. M. Lindsdale. (1937). *Fur-bearing mammals of California*, *volume 1*. University of California Press, Berkeley.

Hall, E.R. (1981). *The Mammals of North America.* 2nd edition. John Wiley and Sons, New York.

Hoffman, R.S., and J.K. Jones, JR. (1970). Influence of late-glacial and post-glacial events on the distribution of the recent mammals of the northern Great Plains. *Pleistocene and Recent environments of the central Great Plains* (W. Dort, Jr., and J.K. Jones, Jr., eds.). *University Press of Kansas, Lawrence*. 355-394.

Hoffmeister, D.F. (1986). *Mammals of Arizona.* University of Arizona Press, Tucson.

Hoofer, S. R., J. R. Choate, and N. E. Mandrak. (1999). Mensural discrimination between *Reithrodontomys megalotis* and *R. montanus* using cranial measurements. *Journal of Mammalogy*. 80, 91-101.

Hooper, E.T., and B.S. Hart. 1962. A synopsis of recent North American microtine rodents. *Miscellaneous Publications of the Museum of Zoology, University of Michigan.* 120, 1-68.

Howell, A. B. (1924). Individual and age variation in *Microtus montanus* Yosemite. Journal of Agricultural Research 28, 977– 1017.

Jones, J. K., Jr., D. M. Armstrong, R. S. Hoffmann, and C. Jones. (1983). *Mammals of the northern Great Plains.* University of Nebraska Press, Lincoln, Nebraska.

Jones, R. T., Knight, R., Martin, A. P. (2010). Bacterial Communities of Disease Vectors Sampled Across Time, Space, and Species. *International Society for Microbial Ecology*, 4, 223-231.

Kendall, D.G. (1977). Diffusion of shape. *Advances in Applied Probability.* 9(3), 428–430.

Lemskaya, N. A., Romanenko, S. A., Golenishchev, F. N., Rubtsova, N. V., Sablina, O. V., Serdukova, N. A., and Graphodatsky, A. S. (2010). Chromosomal evolution of *Arvicolinae* (*Cricetidae, Rodentia*). III. Karyotype relationships of ten *Microtus* species. *Chromosome Research*. 18(4), 459-471.

Liu, H. and A.T. Beckenbach. (1992). Evolution of the mitochindrial cytochrome oxidase II gene among 10 orders of insects. *Molecular Phylogentics and Evolution.* 1(1), 41-52.

Maekawa K. et al. (1999). Molecular phylogeny of orthopteroid insects based on the mitochondrial cytochrome oxidase II gene. *Zoological Science.* 16, 175–184.

McGuire J.L. (2011). Identifying California *Microtus* species using geometric morphometrics documents Quaternary geographic range contractions. *Journal of Mammalogy.* 92(6), 1383-1394.

Moritz, C., J.L. Patton, C.J. Conroy, J.L. Parra, G.C. White, and S.R. Beissinger. (2008). Impact of a century of climate change on small-mammal communities in Yosemite National Park, USA. *Science*. 322, 261-264.

Nagorsen, D. W. (2002). *An identification manual to the small mammals of British Columbia*. British Columbia, Ministry of Sustainable Resource Management.

Randall J.A., R.E. Johnson. (1979). Population densities and habitat occupancy by *Microtus Longicaudus* and *Microtus Montanus*. *Journal of Mammalogy*. 60(1), 217-219.

Sera, W. E. and C. N. Early. (2003). *Microtus montanus*. American Society of Mammalogists, Lawrence, KS. *Mammalian Species.* 716, 1-10.

Smolen, M.J., and B.L. Keller. (1987). *Microtus longicaudus*. *Mammalian Species.* 271, 1-7.

Spaeth P.A. (2009). Morphological convergence and coexistence in three sympatric North American species of *Microtus* (*Rodentia: Arvicolinae*). *Journal of Biogeography.* 36(2), 350-361.

Stangl, F. B.,Jr., J. R. Goetze, and C.B.Carr. (1993). Value of the interorbital breadth in the discrimination of some problematic species ofPeromyscus and Reithrodontomys. *Texas Journal of Science.* 45, 186-187.

Stangl Jr, F.B., J. Goetze, & C.B. Carr. (2004). Historical zoogeography and taxonomic status of the prairie vole (*Microtus Ochogaster*) from the southern planes of Texas and Oklahoma. *Museum of Texas Tech University. Occasional Papers*. 235.

Stewart, J. D. (1978). Mammals of the Trapshoot local fauna, late Pleistocene, of Rooks County, Kansas. *Proceedings of the Nebraska Academy of Science, Abstracts,* 45-46.

Stewart, J. D. (1987). *Latitudinal effects in Wisconsinan mammalian faunas of the plains.* Kansas Geological Survey, Guidebook Series *5*, 153–158.

Sullivan TP, Sullivan DS. (2008). Vole-feeding damage and forest plantation protection: Large-scale application of diversionary food to reduce damage to newly planted trees. *Crop Protection* 27 (3-5), 775-784.

Tuener, R.W. (1974). Mammals of the Black Hills of South Dakota and Wyoming. Museum of Natural History, University of Kansas, *Miscellaneous Publications*. 60, 1-178.

Wallace, S.C. (2001). Confirmations of *Microtus montanus* (mountain vole) from the late-Wisconsinan Jones Local Fauna, Meade Co., Kansas. *Current Research in the Pleistocene* 18, 117-119.

Wilkins, J. S. (2009). *Species: a history of the idea* (Vol. 1). Univ of California Press.

Zeveloff, S.I., and F. R. Collett. (1988). *Mammals of the intermountain west. University of Utah Press,* Salt Lake City.

Table 1 – Cranial measurements and descriptive statistics ($\overline{X}$ = Mean, SE = Standard Error, CV=coefficient of variation) for cranial measurements (in mm) for *Microtus longicaudus* and *Microtus montanus*.

	M. longicaudus						*M. montanus*					
	n	$\bar{X}$	SE	Min-max	Range	CV	n	$\bar{X}$	SE	Min-max	Range	CV
Condylobasal length (CBL)	86	24.92	0.22	20.57-28.32	7.75	8.01	119	23.53	0.23	18.22-28.14	9.92	0.23
Condylobasilar length (CNL)	86	23.95	0.20	19.85-26.9	7.05	7.78	119	22.72	0.21	17.98-26.83	8.85	0.21
Occipital nasal length (ONL)	86	25.45	0.17	21.96-28.03	6.07	6.28	119	23.51	0.19	19.09-27.56	8.47	0.19
Nasal Length (NAL)	86	7.20	0.08	5.72-8.71	2.99	9.69	119	6.31	0.07	4.44-8.08	3.64	12.74
Zygomatic breath (ZYB)	86	14.06	0.11	11.98-16.14	4.16	7.09	119	13.42	0.13	11.08-16.20	5.12	10.43
Mastoidal width (MAW)	86	11.59	.07	10.04-12.95	2.91	5.95	119	10.83	0.08	9.06-13.00	3.94	7.89
Pre-lamboidal breadth (PLB)	86	9.81	0.03	9.31-10.32	1.01	2.45	119	8.77	0.02	7.98-9.40	1.42	3.00
Interorbital constriction (IOB)	86	3.60	0.02	3.04-4.04	1	4.72	119	3.44	0.01	3.14-3.82	0.68	4.13
Upper molar alveolus length (MAL)	86	5.93	.04	5.07-6.75	1.86	6.34	119	5.75	0.04	4.76-6.83	2.07	7.97
Total body length	86	159.34	2.24	99.00-197.00	98	13.01	112	131.48	2.28	83.00-187.00	104	18.35
Tail length	86	55.12	0.89	35.00-75.00	40	14.91	115	34.70	0.64	22.00-55.00	33	19.73
Hind foot length	86	20.57	0.19	18.00-31.00	13	8.71	117	18.11	.22	12.00-30.00	18	13.41

Table 2 – Presence and absence of skull characteristics in *Microtus montanus* and *M. longicaudus*. Fisher's exact test (non-parametric) used with small sample sizes to determine significance in (A.) Presence/absence of temporal groove (B.) Incisor visibility and (C.) Presence/absence of constriction of the incisive foramen. Each test shows that these skull characteristics are significantly different between *Microtus longicaudus and M. montanus* ($p < 0.0001$).

Skull Characteristic	Present	Absent	Indistinguishable	Fisher's exact test p-value
Visibility of incisors, *M. montanus*	95%	5%	0%	<0.0001
Visibility of incisors, *M. longicaudus*	20%	80%	0%	<0.0001
Constricted Incisive Foramen, *M. montanus*	73%	20%	7%	<0.0001
Constricted Incisive Foramen, *M. longicaudus*	12%	79%	9%	<0.0001
Temporal Groove, *M. montanus*	82%	16%	2%	<0.0001
Temporal Groove, *M. longicaudus*	6%	92%	2%	<0.0001

Table 3 – Discriminant function analysis model showing the number of variables included in each model, the number of specimens misclassified, percent misclassified, number of skulls able to be identified by each model (out of 205 specimen) and the -2LogLikelihood.

	External measurements	Skull measurements	External and skull measurements	Best fit measurements (Tail length, mastoidal breadth, prelamboidal breadth)
Number misclassified	9	1	0	0
Percent misclassified	4.569	0.488	0	0
-2LogLikelihood	43.84	8.343	0.14	0.402
Total # of skulls identified	197	205	205	201
Total # of model variables	3	9	12	3

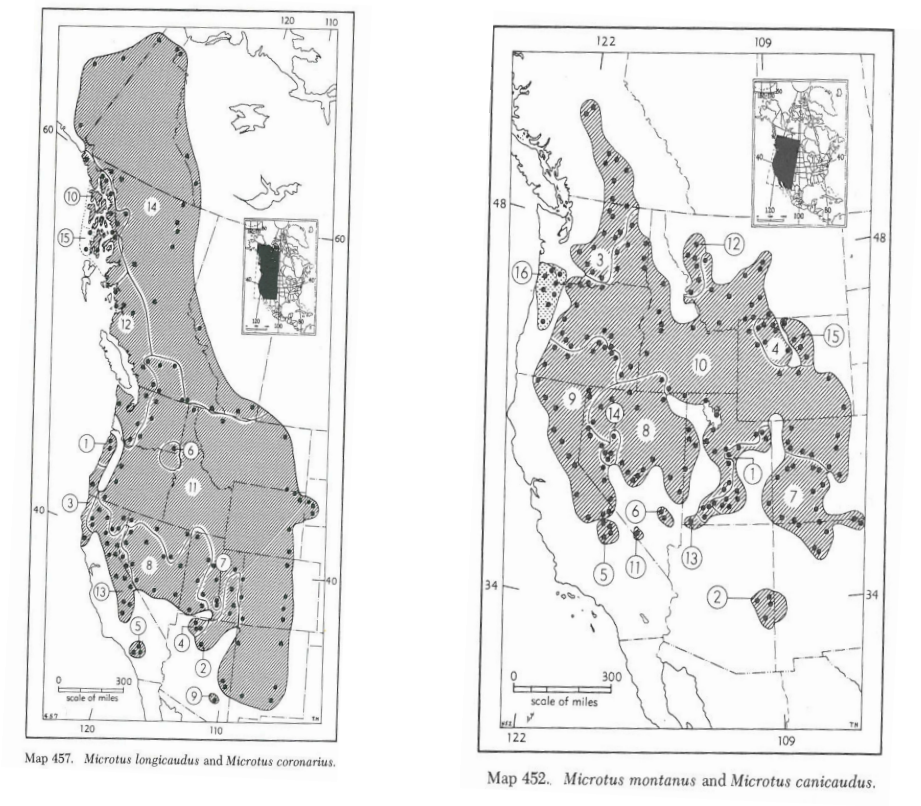


Fig. 1 – (a) Geographic range of *Microtus longicaudus* (left), (b) and *M. montanus* (right) in the North American West and Colorado (modified after Hall, 1981).

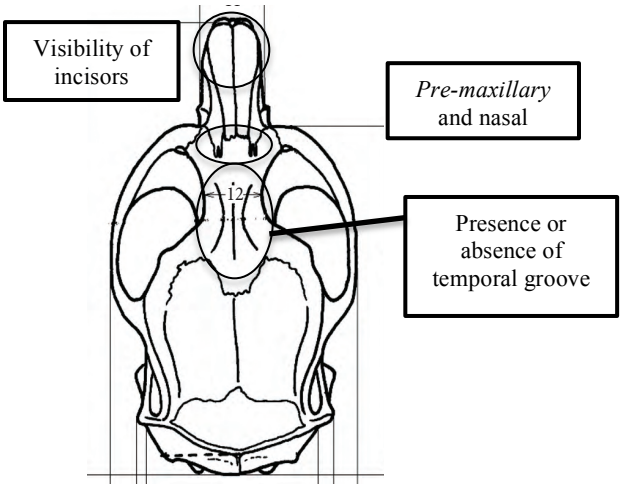


Fig. 2 – Skull characteristics compared between *Microtus montanus and M. longicaudus* as described in Hoofer et al. 1989: Visibility of incisors from above, presence or absences of temporal groove, pre-maxillary and maxillary suture equal or sub-equal. (Modified after Conroy and Gupta, 2001)

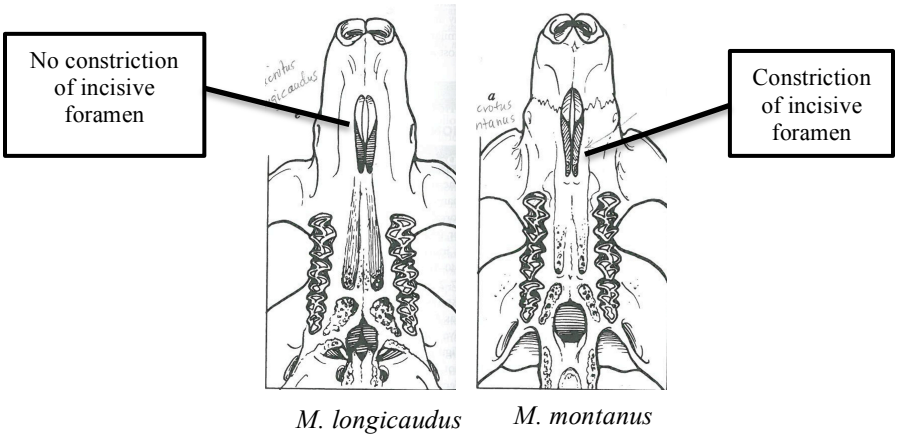
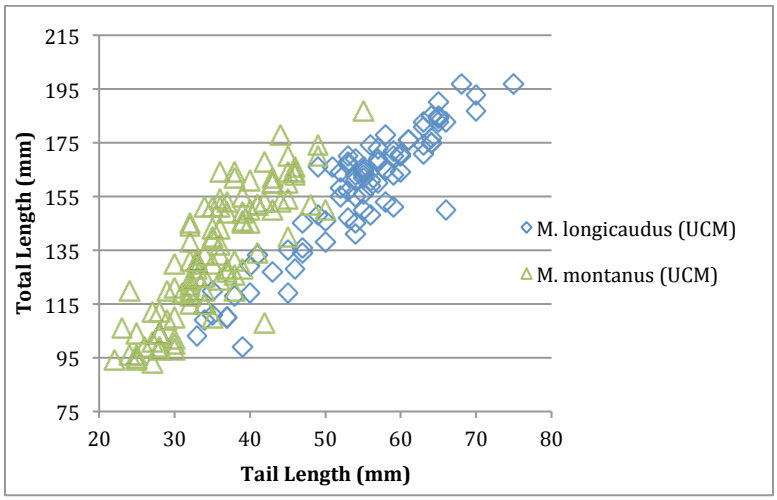
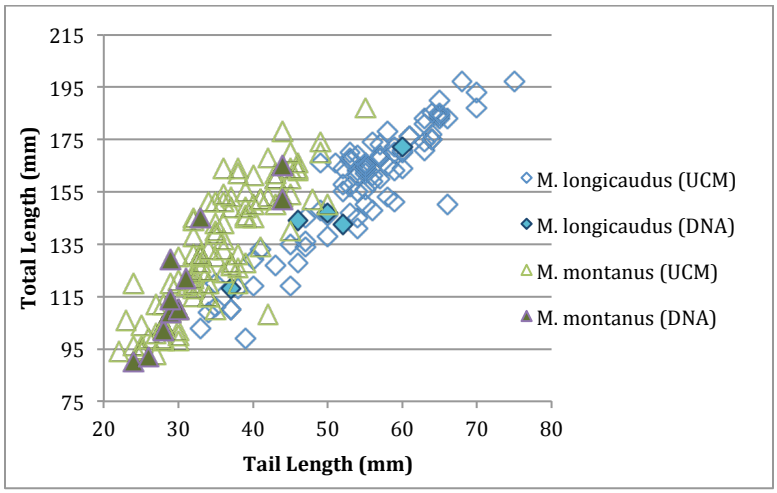


Fig. 3 – Ventral view of *Microtus longicaudus (left)* and *M. montanus* skulls (right). Comparison of the constriction of the incisive foramen as described in Hoofer et al. (1989) and Hall (1981).

a.



b.



c.

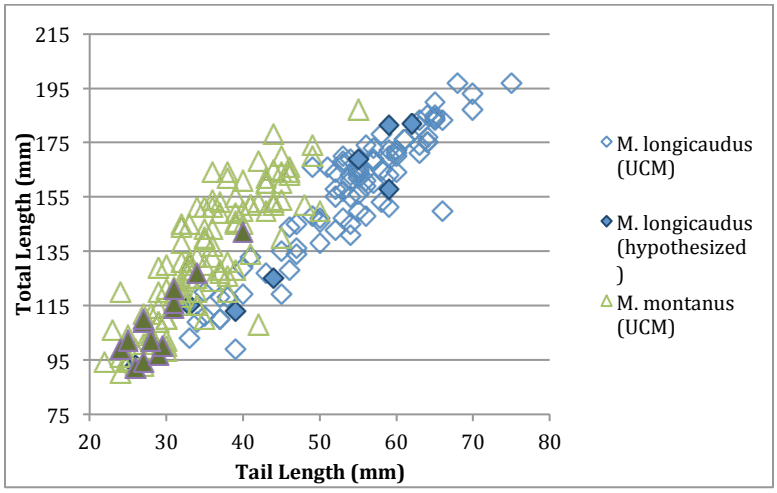


Fig. 4 –Total length of body compared to tail length in *Microtus longicaudus* and *M. montanus*, showing overlap in juveniles in (a) UCM specimen, (b) DNA and (c) specimens of unknown identity.

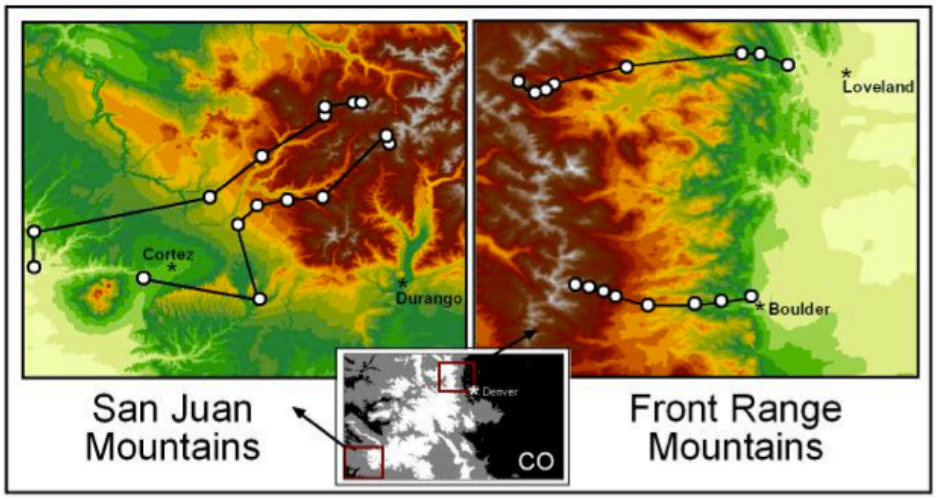


Fig. 5 - San Juan and Front Range Mountain transects where specimens were collected throughout the McCain Studies. (a) The San Juan Mountains included two transect lines with eight sites each (left). (b) The Front Range Mountains included two transect lines with eight sites each (right) (McCain, 2013).

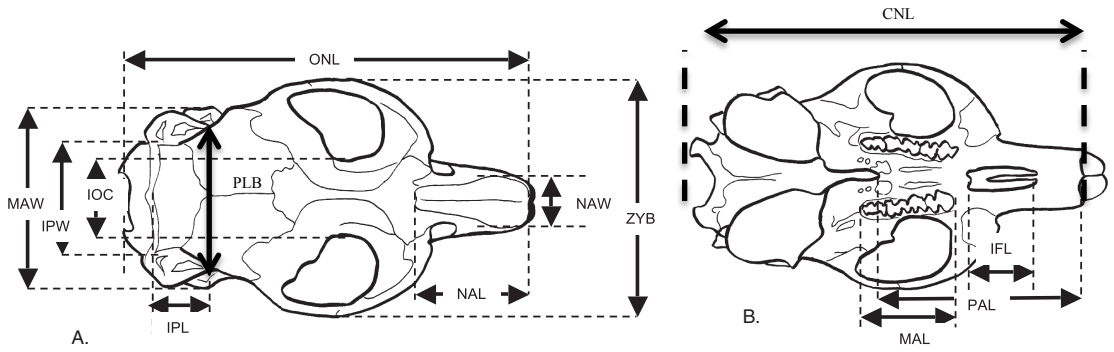


Fig. 6 – Skull with measurements labeled. A, dorsal view. B, ventral view. Condylobasal length (CBL); condylobasilar length (CNL); occipital nasal length (ONL); Nasal length (NAL); zygomatic breadth (ZYB); Mastoid width (MAW); prelamboidal breadth (PLB); interorbital constriction (IOB); upper molar alveolus (MAL). Details of each measurement provided in the text. Modified after Conroy and Gupta (2001).

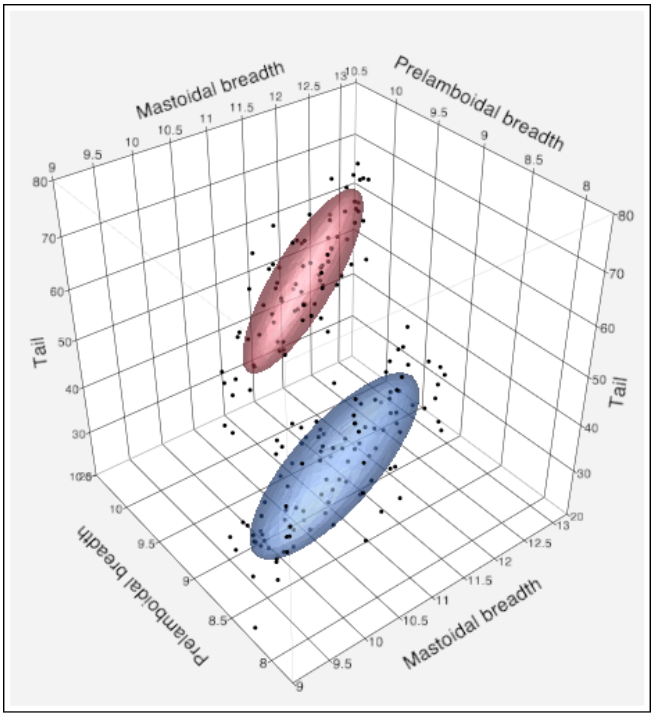


Fig. 7 – Three dimensional model of multivariate analysis of the best fit model using external (tail length) and skull characteristics (mastoidal and prelamboidal breadth) for identifying *Microtus longicaudus* vs. *M. montanus.*

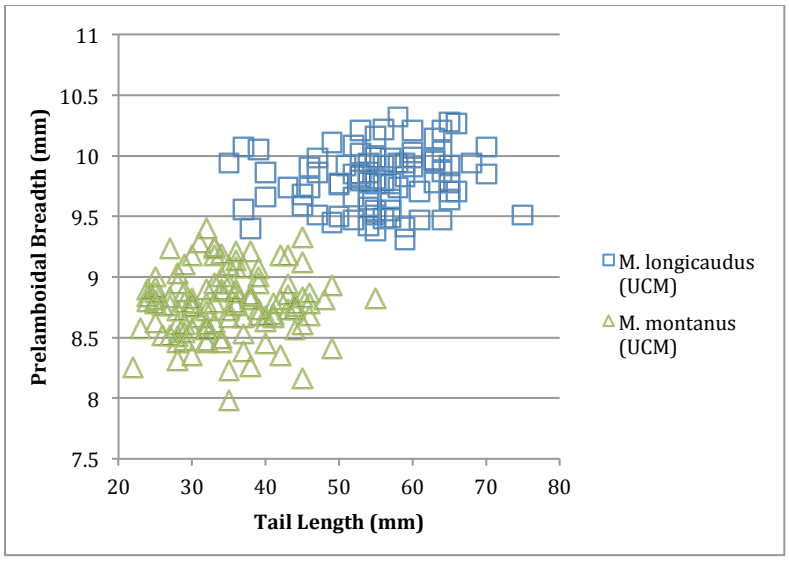


Fig. 8 – A comparison of prelamboidal breadth and tail length (mm) in UCM specimens showing the best bivariate model using skull and external measurements for identifying *Microtus longicaudus* vs. *M. montanus*.

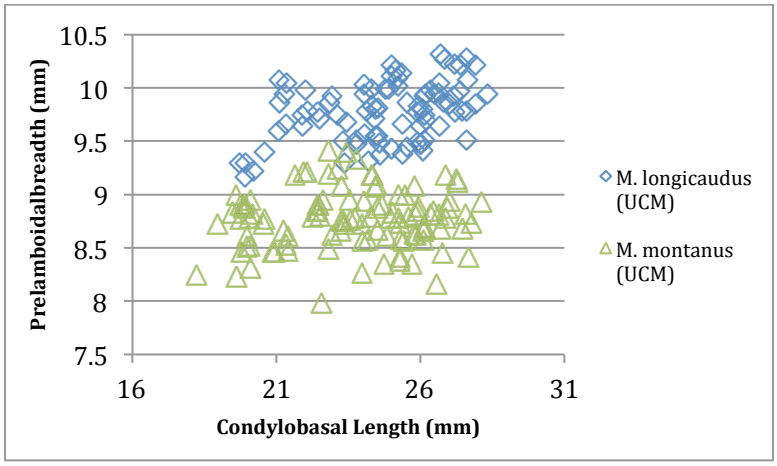
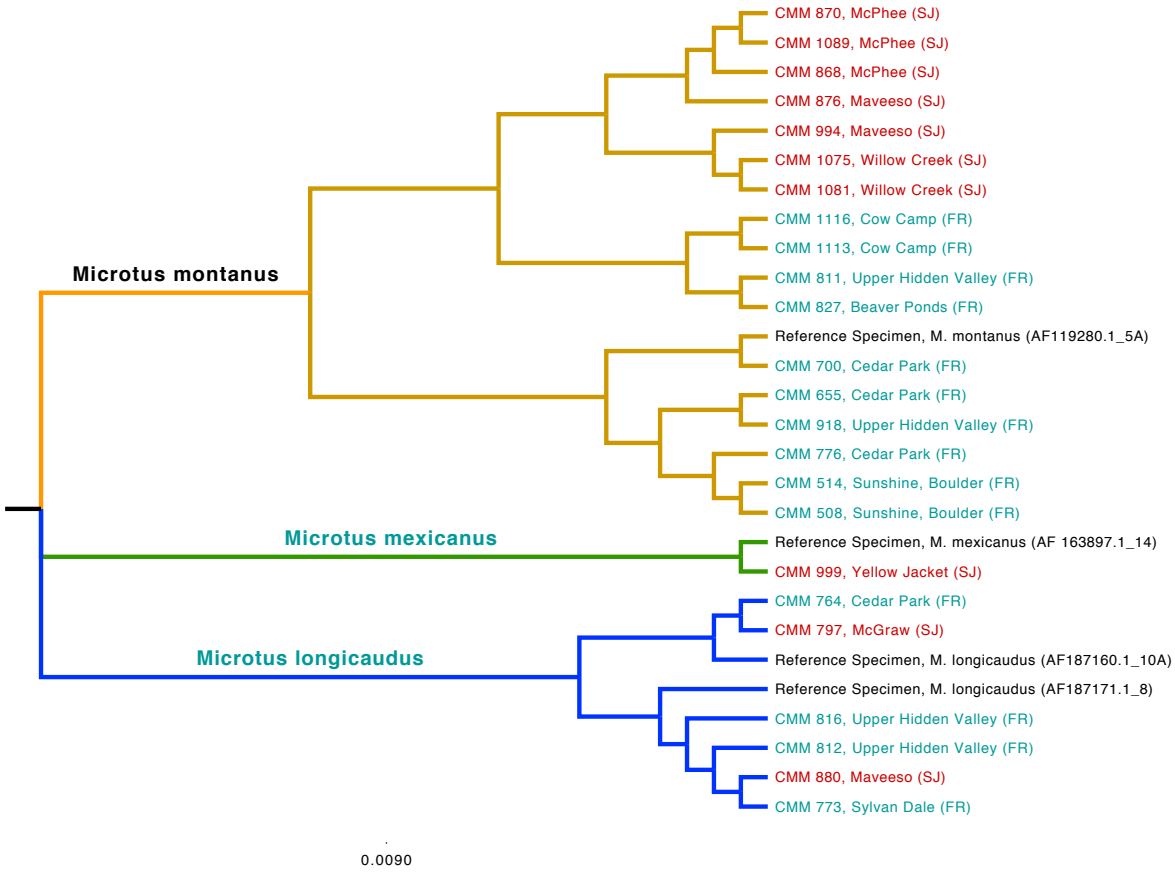


Fig. 9 – A comparison of prelamboidal breadth and condylobasal length (mm) in UCM specimens. showing the best bivariate model using only skull measurements for identifying *Microtus longicaudus* vs. *M. montanus*.

Appendix 1 – Maximum likelihood (ML) tree based on GTR + G + I distances. The tree shows the inferred phylogenetic relationships among 100 cytochrome *b* haplotypes representing three *Microtus* species analyzed from the McCain collection. The length of horizontal branches indicates the number of DNA nucleotide differences that have occurred since the last union between two branches. Specimens in red are from the San Juan mountain range, specimens in green are from the Front Range, and specimens in black are reference specimens obtained from GenBank for comparison.

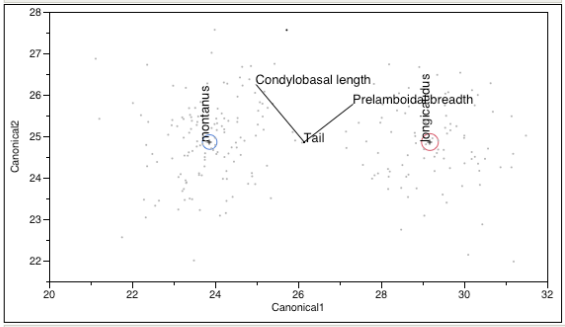


Appendix 2 – Example of multivariate model run on all hypothesized *Microtus longicaudus* and *M. montanus* specimen. This model uses the best fit of tail length, condylobasal length, and prelamboidal breadth. One specimen is misclassified (highlighted) and it is possible to see that the -LogProb of this specimen is above 1.000.

Discriminant Analysis

Discriminant Method: Linear

Canonical Plot



Discriminant Scores

Columns
Condylobasal length
Prelamboidal breadth
Tail

	Training
Number Misclassified	1
Percent Misclassified	0.439
-2LogLikelihood	9.106

Row	Actual	SqDist(Actual)	Prob(Actual)	-Log(Prob)		Predicted	Prob(Pred)	Others
1	longicaudus	0.34013	1.0000	0.000		longicaudus	1.0000	
2	longicaudus	0.83223	1.0000	0.000		longicaudus	1.0000	
3	longicaudus	1.51335	1.0000	0.000		longicaudus	1.0000	
4	longicaudus	5.65565	0.9932	0.007		longicaudus	0.9932	
5	longicaudus	0.30455	1.0000	0.000		longicaudus	1.0000	
6	longicaudus	1.11297	1.0000	0.000		longicaudus	1.0000	
205	montanus	0.48878	1.0000	0.000		montanus	1.0000	
206	montanus	3.20711	1.0000	0.000		montanus	1.0000	
207	longicaudus	3.29911	1.0000	0.000		longicaudus	1.0000	
208	montanus	6.60997	0.9976	0.002		montanus	0.9976	
209	montanus	1.32587	1.0000	0.000		montanus	1.0000	
210	longicaudus	20.37540	0.0143	4.246	*	montanus	0.9857	
211	montanus	0.61190	1.0000	0.000		montanus	1.0000	
212		.	.	.			.	
213	montanus	1.03153	1.0000	0.000		montanus	1.0000	
214	montanus	2.21947	1.0000	0.000		montanus	1.0000	
215	montanus	1.81367	1.0000	0.000		montanus	1.0000	
216	longicaudus	0.25666	1.0000	0.000		longicaudus	1.0000	
217	longicaudus	1.44339	1.0000	0.000		longicaudus	1.0000	
218	longicaudus	1.47877	1.0000	0.000		longicaudus	1.0000	
219	longicaudus	6.41943	0.9960	0.004		longicaudus	0.9960	
220		.	.	.			.	
221	montanus	4.91649	0.9993	0.001		montanus	0.9993	
222	montanus	4.80723	0.9993	0.001		montanus	0.9993	
223	montanus	1.83921	1.0000	0.000		montanus	1.0000	
224	montanus	1.39956	1.0000	0.000		montanus	1.0000	
225	montanus	0.22629	1.0000	0.000		montanus	1.0000	
226	longicaudus	3.78710	0.9991	0.001		longicaudus	0.9991	
227	montanus	4.21761	0.9999	0.000		montanus	0.9999	
228	montanus	0.69767	1.0000	0.000		montanus	1.0000	
229	montanus	1.03170	1.0000	0.000		montanus	1.0000	
230	montanus	0.50233	1.0000	0.000		montanus	1.0000	
231	montanus	0.94110	1.0000	0.000		montanus	1.0000	
232	montanus	3.31381	0.9999	0.000		montanus	0.9999	
233	montanus	3.27638	1.0000	0.000		montanus	1.0000	
234	longicaudus	8.44929	0.9680	0.033		longicaudus	0.9680	

'*' indicates misclassified

Training

Counts: Actual Rows by Predicted Columns

	longicaudus	montanus
longicaudus	93	1
montanus	0	134