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24 March 2004

TO: Rob Roy Ramey III and Gary Skiba

FROM: David M. Armstrong

RE: Ramey *et al.* Report on *Zapus hudsonius preblei*

What follows are observations on a report by Ramey, Liu and Carpenter (2004) entitled "Testing the Taxonomic Validity of Preble's Meadow Jumping Mouse (*Zapus hudsonius preblei*)" dated 12 March 2004.

I have taken literally the invitation of the senior author (by telephone and on p. 15 of the Report) to provide comments and constructive criticisms; that has been my intent. I trust that the comments to follow will be taken in the collegial spirit with which they are offered. Obviously, as with any peer review, a little country wisdom may be salutary: consider the source, remember how much it cost you, and take it or leave it.

Caveat lector: my comments may reveal a some annoyance or otherwise appear to be hypercritical. I believe that this is annoyance with the process and not with the substance of the report or its authors.

The background is this: I have a fair amount of experience (> 35 years) providing reviews of technical manuscripts for professional journals. In that process one presumes that comments can be considered and if appropriate and relevant can be incorporated to improve a paper. The "industry standard" is either an informal, collegial "preview," before a manuscript is finalized and submitted for publication, or it's a collegial peer review, at the request of a professional journal, intended to fine-tune a submitted paper and/or to recommend for or against publication.

You'll find that I continue to suggest changes in spelling, typography, grammar, and the like although I realize that this report is *fait accompli.*

In the present process, the report apparently has been submitted and publicized, so it is beyond the point of useful, collegial, constructive input. Still, I will make editorial comments as well as substantive comments (1) because I can't help myself and (2) because the two are not unrelated. The medium is part of the message. Further, the comments may be useful as this report is re-written as a manuscript for possible publication.

For what it's worth, I had the same trouble reviewing (at the request of the USF&WS) various notices in the *Federal Register* concerning *Zapus hudsonius preblei*; I had the conflicted feeling that, on one hand, I wanted to do the right thing for colleagues or the mice, but—on the other hand—from any practical standpoint the review was irrelevant. In that case, I did the "reviews" anyhow

because they were required by some bureaucratic and political process. In the present case I decided to do the reviews anyhow because they were requested by friends and I suppose there is the chance that they will influence an eventual published paper on this topic or eventual conservation success in local and regional ecosystems.

The substantive, take-home message from the report is that the authors performed sophisticated molecular genetic studies, tested statistically some *a priori* hypotheses, conducted a multivariate statistical assessment of the quantitative measurements used by Krutzsch (1954), and evaluated the logic of qualitative descriptions and comparisons in Krutzsch's original description of *Zapus hudsonius preblei*. They concluded that the supposed subspecies is not worthy of subspecific recognition, by standards promulgated in part by the senior author in a cited paper. Further, they concluded that the population of *Zapus hudsonius* of central Colorado and adjacent southeastern Wyoming is not worthy of conservation attention.

These are interesting and important conclusions, important to a variety of human stakeholders (including—but not limited to—scientific, management, legal, political, and economic interests) and also to a peripheral and apparently disjunct population of meadow jumping mice.

In addition to the conclusions, there are some lingering questions implicit in the report, the answers to which should be critical to policy-makers. Specifically, the report mentions but does not explore in depth the possibility of hybridization between *Zapus hudsonius* and *Zapus princeps*. Depending on the nature and extent of that hybridization (which is not detailed), taxonomists might wish to re-evaluate the validity of *Zapus princeps* as a species. Also, the report suggests the possibility that *Zapus hudsonius preblei* is a peripheral isolate, but does not explore the possible implications of a disjunct range in any particular depth.

Turning to general comments on the report *per se*, I suspect that part of the problem I have with the report may be simply that it is, in fact, a report, not a manuscript intended for publication. A manuscript intended for actual publication would be double-spaced, with room for editorial remarks. A manuscript intended for publication probably would reflect or represent the usual style or tone of the systematic literature. This report, by contrast, is sometimes repetitive, argumentative, and dismissive of the methods of at least one systematist of an older generation; excursions into "the scientific method" and pleading about standards of critical thinking have no place in a professional taxonomic article (although they might be appropriate in a philosophical or methodological essay). A taxonomist does not have to talk about critical thinking; s/he just has to do it.

I am not competent to provide peer review of the methods and procedures of molecular systematics. Although I will raise some questions about the assumptions of the analysis and conclusions, my comments are mostly confined

to taxonomic issues (both substantive and "stylistic") and geographic issues and timescales—especially lingering questions.

By the way, in a report addressed (and by now submitted) to a high elected official and a Federal agency I would have expected a very high standard of presentation. Therefore I was surprised by the number of grammatical and typographic errors, and the general laxity of style (it not being obvious with which, if any, professional stylesheet the report was intended to conform).

I have made some editorial remarks on the report itself, as agreed with Ramey. Minor suggestions are made directly on the report. More extensive or substantive remarks are listed below, keyed to marginal numerals on the report.

Thanks for the opportunity to look at this report. I do hope that some of my comments will be of value. If there are questions about specifics, let me know. E-Mail would be the most expeditious means of communications; to increase the probability of timely response, please address both mausmann@aol.com and david.armstrong@colorado.edu.

1. Given the quotation marks, the phrase "unsupported opinion" feels like a term of art and must stem from some source with which I am not familiar. I found the use of this phrase within quotation marks distracting. Would it not be more informative simply to say that qualitative characters used by Krutzsch (1954) apparently represent erroneous observations or extrapolation from too few specimens and are not supported by examination of the larger samples now available?

2. Where is the controversy? Cite some literature to indicate the history. Who said what when and where? Citation of representative papers from the taxonomic (or other) literature on either side of the allegedly controversial issue would seem like minimal requisite justification for the statement. If this is only a political or administrative controversy, and not a taxonomic one, that should be explicit. Again, citation is critical, to give credit where due and to allow readers the opportunity to confirm (or deny) assertions.

3. We learn only later what those "modern standards" are. Out of fairness to the intellectual process and to the spirit of science as a cumulative, self-correcting enterprise, if it really is necessary to judge Krutzsch as a scientist, here and elsewhere, he should be judged by the standards of 1954, not those of today. That is not to say that his conclusions cannot be re-evaluated and perhaps rejected, based on new techniques, newly available specimens, or even re-examination of old specimens. Errors are errors, and if they are identified they certainly should be corrected. Old Darwin was right (I paraphrase): false facts [i. e., errors of observation] are highly injurious to the progress of science, for they often endure long, but false theories [and a taxonomic judgment is an hypothesis or a theory, after all] are beneficial to science because everyone takes such salutary pleasure in proving them wrong.

4. Is this statement consistent with the alleged controversy noted earlier in this paragraph? If the taxonomy was not questioned critically by the scientific community, then where was or is the supposed controversy?

5. In the spirit of giving clear credit where due, I would note that Krutzsch (1954) proposed that *Zapus hudsonius* is subdivisible into 11 subspecies, two of which (*Z. h. preblei* and *Z. h. intermedius*) were named by Krutzsch himself. The 12th subspecies, *Z. h. luteus,* was recognized by Hafner *et al.* (1981) as representing *Z. hudsonius* rather than *Z. princeps*, with which it had been arrayed by Krutzsch). I would cite Hall (1981) as a more recent source of a map of the geographic range of *Z. hudsonius*. I believe that is the most recent such map based on examination of all of the literature; if I recall, Whitaker's (1972) map was based on Hall and Kelson (1959).

6. I think the statement that "The range of *Z. h. preblei* is restricted to the base of the Front Range in Colorado and into southeastern Wyoming" is equivocal. What does "the base of the Front Range" mean? In popular parlance, the "Front Range" seems to be the I-25 corridor. To a physiographer, the Colorado Front Range is that easternmost range of the Southern Rockies, extending from Pikes Peak north to the Poudre River. *Z. hudsonius* apparently ranges north beyond the Colorado Front Range in the "Laramie Foothills" and along the front of the Laramie Mountains of Wyoming as well.

7. I question the phrase, "the presumed cause of its uniqueness is the retreat of moist riparian habitat." If the population is distinct, then the cause of uniqueness is not habitat change but changes in gene frequencies, presumably due to natural selection. This needs to be clarified. Is the statement about proximate cause, ultimate cause, direct environmental influence, or what?

   By the way does this sentence imply that the populations now known as *Z. h. preblei* are geographically isolated from the conterminous range of the species? I think that is an important consideration, from standpoints of both evolutionary genetics and conservation.

8. What is the relevance of this statement? Perhaps the intent is to underscore the need for the present study. The facts are that although Connor and Shenk (2003) used discriminant analysis of cranial measurements to distinguish *Z. hudsonius* from *Z. princeps*, to date there has been no thorough study infraspecific variation of *Z. hudsonius* in Colorado and adjacent Wyoming, and no modern study (at the level of either morphology or molecules) comparing *Z. h. preblei* with other named populations of meadow jumping mice.

9. I find this confusing. If *Zapus hudsonius* is found to "freely hybridize" with *Z. princeps*, then *Z. hudsonius* is not a separate species (or *Z. h. preblei* has been allocated to the wrong species). If hybridization exists but is less than "free," then more subtle analysis would be called for. Either way, this statement raises major, unanswered questions.

10. I think that one can test genetic distinctiveness but not "taxonomic validity." (Also see my suggested changes to the title.) In the end, "taxonomic validity" is a judgment call, made by the community of systematic biologists in a dynamic, iterative, self-correcting process. A taxonomist makes a judgment, and other specialists either do or do not follow her or his arguments.

The first level of this process is peer review of a manuscript. Then the manuscript is published and becomes part of the formal record of science.

Even having been published, a taxonomic assertion is not an absolute but a sort of trial balloon, a testable hypothesis if you will, which may or may not be generally accepted in the long run.

In practice, for most groups of organisms, the taxonomic assertions of one author will remain the standard until another researcher revises the taxon in question. Sometimes this standard persists for many decades. Krutzsch (1954) is such a standard. It will remain the basic reference on North American "zapodids" until someone re-examines the material available to Krutzsch, adds to the analysis all of the material (or a statistically reliable sample thereof) that has accumulated since that time, adds an analysis of characters not available to Krutzsch (molecular data for example), and reaches new conclusions.

11. This sentence may include too many thoughts to be readily understandable. You used modern methods of genetic and phylogenetic analysis AND you used modern concepts of subspecies and distinct population segments. These are two quite different things and I would suggest that they be kept separate. You are using new techniques and you are also suggesting a change in the standard, in effect, raising the bar. New techniques can lead to new biological insights; that's great, I think. New standards represent a new judgment call. This is not quite biology, I think, but a matter of taste.

    In the context of standards, I keep remembering an old but sage piece of advice: "...a subspecies should be described only when to fail to do so would obscure more biological truths than would be lost by describing the subspecies" (*in* Jameson, D. L., *et al.* 1966. Proc. California Acad. Sci., 4th Ser., 33:551-620).

12. This is a personal statement about a personal reaction. I get uncomfortable when I hear comparative biologists talking about "the scientific method," as if there were just one way of doing science. It sounds pretentious, for one thing, so may put off some readers. But more important, some philosophers of science would argue that comparative biology (in which phylogenetic analysis is included) cannot be scientific because it does not permit real experiments. Individuals and species are unique genetic entities at unique points in space and unique moments in time; they are not replicable, by definition.

    This does not mean, of course, that a person cannot pursue phylogenetic analysis rigorously, honestly, and productively. I just would not glorify that

as "*the* scientific method." Tone down the rhetoric and avoid the unavoidable and necessarily fruitless philosophical discussion.

13. Does this rule-of-thumb provide a basis to put real time into some statements later on that are presently ill-defined? (*e. g.*, comment #26 keyed to Report, p. 9

14. Here and throughout, this quasi-formal notation of hypothesis-testing may be lost on a lay audience. Quotation from the proposal feels like overkill to me. In a report (although perhaps not in a journal, with its stylistic standards), one could use layout techniques like indentations, boxes, or even boldface to set these off from the general text.

    By the way, this insistence on rigorous hypothesis-testing (however laudable in itself) is also "bait" for attorneys who may miss the zoology if drawn too strongly to the niceties of the argument.

15. I realize that this terminology is used in a variety of publications (official US Fish & Wildlife Service petitions, for example). But as a matter of convention I would definitely avoid using "Preble's" as if were the name of something. Since about the 1930s most mammalogists have not given vernacular names for most subspecies, except for game species like desert bighorns and Roosevelt elk. So I would not even say "Preble's meadow jumping mouse"; I would say *Z. h. preblei.*

    I would never, ever say "Preble's"; that is a possessive, an adjective. An adjective without a noun is meaningless. One would never say "that's a big" and expect it to convey meaning. Even if mammalogists did use vernacular names for subspecies, "Preble's" is equivocal. Do the authors mean *Sorex preblei, Tamiasciurus hudsonicus preblei, Dipodomys microps preblei, Peromyscus truei preblei, Phenacomys preblei,* or *Lutra canadensis preblei*?

    Now I realize that the meaning is clear from context, but that's not the point. The point is, this usage is not idiomatic "mammalogese," so it might well sound un-professional to the wrong ears.

16. What does this have to do with the central question of this report, the taxonomic status of *Z. h. preblei*?

    The observation of possible hybridization raises the important and interesting question of whether or not *Z. princeps* J. A. Allen (1893) is a valid species, distinct from the earlier-named *Z. hudsonius* (Zimmermann, 1780). However, at this point in the report it does seem to me like a secondary consideration, perhaps deserving of a little more play but in a separate paragraph. Hybridization with *Z. princeps* has been raised as a

possibility by various authors and certainly deserves to be explored, but that is an issue separate from variation within a putative subspecies, and, of course, those who have suggested the possibility of hybridization should be cited.

17. Good. Does this method also raise the question of geographical continuity? I think this may be a critical consideration with *Z. h. preblei*, and I know of no published study to try to understand the degree to which *Zapus hudsonius* is more or less continuously distributed (as a metapopulation) across eastern Wyoming, connecting the range of *Z. h. preblei* with that of either *Z. h. pallidus* or *Z. h. campestris*.

18. I agree with the importance of tying molecular samples to museum specimens. I agree strongly with the value of museums as repositories of information. However, I also am disappointed that ear punch samples could not be included in some part of this study. Basing this analysis on the few available museum specimens greatly restricts its utility. Under USF&WS protocols during the "PMJM Campaign," specimens could be prepared only of inadvertent trap casualties; however, my understanding is that a fair number of ear-punch samples were taken at the direction of USF&WS and CDOW. I presume that these were tied with specific geographic localities. Being conservative, I assume that field identifications of species of jumping mice are to some extent unreliable. However, using modern statistical techniques, I presume that one could run all of the available tissues and use some kind of discriminant analysis to sort *Z. hudsonius* from *Z. princeps*—assuming that there are genetic differences. This material could also be quite useful in approaching the separate but fundamental question of hybridization between *Z. hudsonius* and *Z. princeps*. Further, I suspect that the ear-punch samples could expand the geographic range of the analysis, which could only be a benefit to the reliability of the study.

19. It feels like this suspicion could have been tested. It could be a useful contribution to the collections management literature. I would be surprised to learn that specimens as late as 1980—especially those preserved in the semi-arid West—had arsenic in them. I prepared a few thousand specimens before 1980 and none of them was "preserved" with arsenic. If there's a chemical culprit interfering with amplification, I wonder if maybe it is residual organics from the bad old days (extending beyond 1980 even to the present in some collections) when insect repellants (Vapona®, PDB) or toxicants (CS_2) were routinely used in collections.

20. How do we know the "most closely related subspecies to *Z. h. preblei*"? Wouldn't the conservative statement be something about the geographically nearest neighboring populations?

21. This needs clarification and more extensive justification; this is an important assertion. The implication is that these specimens represent *Z. princeps*. Admittedly, I have not worked in that area for a number of years and I may be behind on the distributional literature, but to my knowledge, *Z. princeps* has not been reported from either Carter County, Montana, or Custer County, South Dakota. I'd dig deeper here. This could be a big deal.

22. This is an interesting result, but most stylesheets probably would not allow the underlined statement for emphasis. If there is concern that this assertion might get lost in the detail, I would set it off as a separate, short paragraph to draw the reader's eye to it.

23. First, this feels like a matter for Discussion rather than Results. Back in my editorial days, the rule-of-thumb was that if authors need to cite literature, their statement is Discussion, not Results. Moreover, when this is moved down the page to the Discussion, it deserves more explication. Explain to the reader how one might have a founder effect—or even an expanding range—without having some restricted genetic exchange at some level in the population.

    Also, this section raises again my query #17, above, about continuity of range across eastern Wyoming to contact either of two possibly contiguous subspecies, *Z. h. pallidus* in the Platte River drainage, or *Z. h. campestris* in the Black Hills (*sensu lato*).

24. Is lower genetic variability not consistent with isolation and a founder effect?

25. It might be wise to review the assertion (from p. 5) that "recent" is intended to mean within the past 10,000 years and "very recent" is intended to mean within the past few hundred years. Also, I'm not sure what "few" means, but it might be important. If "a few" is 300-400, for example EuroAmerican influence would have come to roost in the range of *Z. h. preblei*; if it's > 300-400, then one would want to think about other external causes that could have influenced the distribution of meadow jumping mice (to test against the null hypothesis of random!)

    Also, speaking as a biogeographer (not as a molecular systematist) I wonder why the insistence on comparison and connection with *Z. h. campestris* rather than *Z. h. pallidus*. Based on simple geography, one might reasonably suspect that mesic-adapted mammals of the South Platte River drainage (which includes most of the range of *Z. h. preblei*) would be more likely to be ecologically and genetically continuous with conspecifics of the Platte River drainage than with those to the north in the

Cheyenne and White River watersheds and beyond. If the genes say otherwise, so be it, but the geography deserves mention.

26. What does "long-term resident" mean here in the context of previously used, somewhat more explicit timeframes, "recent" and "very recent"?

27. Here and elsewhere, I wonder what ecological evidence for divergence would look like? I would think that a much more important criterion for recognizing a population as distinctive would be geographic isolation and lack of (or greatly restricted) gene flow. This report presents some evidence that there is lack of gene flow, but does not address the question of geographic isolation. Have there not been studies in southeastern and east-central Wyoming to address this issue at least in broad terms? As a rough first approximation, where have folks looked for meadow jumping mice, following established USF&WS protocols, and not found them? (I realize that lack of captures does not prove that the mice are not present, but simply that they were not caught, but still those trapping results are a start.)

28. A couple of points are stimulated by this paragraph. First, an agency report is not a publication and is therefore an inappropriate place to synonymize two subspecies. This report is private communication , or at most "gray literature," and it has no standing in zoological taxonomy. Second, the report seems not to recognize that this is a fairly small piece of the puzzle of geographic variation in the meadow jumping mouse. Absent a thorough taxonomic revision of subspecies of *Zapus hudsonius*, for example, I am not sure why *Z. h. preblei* should be considered a synonym of *Z. h. campestris* and not of *Z. h. pallidus*. And the authors have not investigated the distinctiveness of either *Z. h. pallidus* or *Z. h. campestris* relative to *Z. h. intermedius*, which is ascribed a range downstream in the Missouri-Mississippi watershed. And so forth.

    In other words, a restricted, targeted investigation of this kind, laid out in an unpublished report, is not an appropriate vehicle for a taxonomic decision of the kind proposed. Rather, changes in infraspecific taxonomy and nomenclature should be based on thorough restudy of the species across its range—in other words, a study on the scale of Krutzsch (1954), but using the methods developed over the past 30-50 years to allow new and more sophisticated insights into evolutionary and ecological processes that any taxonomy ought to reflect. And they must be published in the peer-reviewed literature.

    This is not to say, of course, that genetic answers to some narrow might not influence the choices that managers (depending upon the latitude allowed them by prevailing laws and regulations).

29. I could not follow the argument here (or at least, in the absence of knowledge of reproductive continuity between *Z. h. preblei* and other populations of *Zapus hudsonius*, I cannot evaluate it). My simple guess would be that if a population is not only peripheral but isolated, then it can—given time—have its own distinctive evolutionary role and tendencies. Therefore, my own simple (ethical, not demonstrably "scientific") conclusion is that it ought to have conservation attention.

    This leaves me wondering what the law and subsequent regulations have to say in this matter. I will leave that to others. My question is, does it make any difference that the population is disjunct even though it is not—yet—distinct by the criteria that have been utilized in this report?

30. This gratuitous indictment of Krutzsch (1954) is not useful. Of course his work is representative of mid-20th Century systematics. Notice when it was published! In fact, one could go back to W. H. Osgood's (1909) masterful revision of mice of the genus *Peromyscus* and assert that Krutzsch's work is merely an extension of methods now nearly 100 years old. That observation would be equally true and equally misplaced. What systematists of the last two to three generations have done was to move beyond the pre-Darwinian, typological systematics of their own predecessors, just as the thoroughly modern systematists of today can—with appropriate diligence and nifty chemical and statistical analyses — move beyond the skin-and-skull taxonomy of their intellectual parents and grandparents. That kind of change is to be expected and to be encouraged, but the change can (and, I would opine, should) be made without demeaning one's predecessors.

    Do not misunderstand. If Krutzsch made mistakes in measurements or interpretations, they should be corrected. If his sample sizes were too small to accurately represent variability within a population, we can do a better job today and we should.

    Old Isaac Newton was not your most humble of scientists, but even he was reported to have said (paraphrasing predecessors of his own) "if I have seen farther it is because I stand on the shoulders of giants." In science as in real life, I think a little humility goes a long way. Obviously, that is a statement of taste and manners, of course, not science.

31. A personal observation: I agree that it would be wonderful if there could be a modern systematic revision (based on phylogeographic analysis of molecular and morphological data) of all of the taxa that might be proposed for listing under the ESA. To be relevant, these would need to be on the scope of thorough revisions of whole species, not just the peripheral or disjunct populations that tend to populate the list of endangered species (and subspecies). I have not taken time to guess the

numbers of taxa that would need to be evaluated but I suspect that the total enterprise (even at the rate of $57k per subspecies) would be prohibitively expensive. I do note that Hall (1981), listed 3607 subspecies and monotypic species of mammals in North America. Perhaps half of those are in the USA, and one would expect similar numbers of lissamphibians, squamate reptiles, and perhaps twice that many kinds of birds and even more kinds of fishes—and that considers only vertebrates, of course, not the whole biota. The total cost could exceed $1 billion.

Beyond the financial cost, there is a huge opportunity cost. Conservation delayed is conservation denied. I happen to agree with the authors that there is room for improvement in the ESA and the consequent regulations and procedures (and even habits of mind) of the USF&WS and its personnel. I think we should move beyond endangered species (or subspecies or other evolutionarily significant units) to consider an Endangered Ecoregions Act (because the integrity of most ecoregions in the US is threatened or endangered) and then move internationally to an Endangered Ecosphere Treaty. But I digress; these are supposed to be comments on a report on a particular subpopulation of meadow jumping mouse!

32. I agree with the general assertion here. But (see above) I am not sure such a report is the place to take on all of the opportunities to improve the Endangered Species Act and its administration, however.

33. I do not recall having any substantive conversation or correspondence about this project prior to the preparation of this report, either at first or second hand. Certainly I do not deserve or desire any acknowledgment. My name (and that of anyone else on this list who had as little to do with the study or report as I did) should be removed from the list. This is a simple matter of giving credit where due and not giving credit where it is not due.

34. The word "catalog" has technical meaning to a field collector or a museum curator. This is not a catalog, but a list. A list of "specimens examined" is a typical feature of a taxonomic paper. However, this is not a conventional list of specimens examined (which do take a fairly wide range of formats).

    Here specimens are listed in the order they were examined (measured?); conventionally, specimens would be listed by locality, in some geographical order. Sometimes this order is alphabetical by county within states ordered alphabetically.

    Old Joseph Grinnell (followed by the late E. R. Hall and some of Grinnell's other students and their students, etc.) went a step farther. He urged that we arrange specimens in the museum geographically, from north to south

and west to east. This is an elegant system because it allows one to look in a tray of specimens, or several trays of specimens laid out side-by-side, and possibly get a first impression of geographic variation in size and color. That Grinnellian convention has been followed in lists of specimens examined by some authors, and I would recommend it. It urges a geographic dimension that is important in any discussion of subspecies because subspecies—whatever their value or quantitative definition—are fundamentally geographic and genetic concepts They are geographically continuous subdivisions of species.

Usually in a manuscript for publication or a technical report, appendices appear after other end-matter (figures, tables).

35. By convention, in most stylesheets, figure legends go beneath figures. Also, and more important, I note that Figure 1 was run in with text on p. 6, whereas this Figure 2 is at the end of the report, buried behind an appendix. In a manuscript for publication, tables, figures, and appendices all would be placed at the end of the manuscript. In a report, I probably would put figures and tables in place in text, in part because that is where lay readers would expect to find them. The end of the report seems curious placement for Figure 2, which presents the data at the heart of the argument.

36. I wonder if these data belong in a table (which, in a report, I would run in with the text for ease of availability) or in an appendix (which does belong back here in the end-matter). This feels like it might be a worthy appendix, but authors might feel this is more important to the argument than that. (Appendices tend to be ignored by all but the most earnest readers.)

More important, I found myself wondering if there were not some way to map these data. Because subspecies are geographic ("mappable") entities, the argument in this paper should be about the geography of evolution. However, geography is difficult to find in the report except by implication, and almost never in detail. Mapping the distribution of the distinctive sequences would help me (and perhaps other readers) to visualize any geographic pattern that might exist. In other words, I would appreciate a phylogeographic analysis and discussion.

Further, because the intent of this paper is to test the validity of the taxonomic concept *Z. h. preblei*, I would definitely call these "supposed subspecies," so that the reader is reminded of the fact that there is some question about their validity.

Here and elsewhere, I find listing specimens examined only to county inadequate. Some of the counties mentioned are larger than some eastern

states. Particularly in the absence of a map, some sense for the geography of the situation is critical.

In this table and Table 2, I noted two different misspellings of "*preblei.*"

Also, the column heading "Subspecies as per museum tag" caught my eye. The name on a museum tag has no standing in a taxonomic work; the assumption in a taxonomic work is that the person who examined the specimen determined the identity of ("expertized") the specimen.

37. Here and in accompanying text, emphasis is on Krutzsch (1954) and not on mice. It's fine to point out inconsistencies in the original description, but it seems to me much more important to re-examine the mice, with the greatly expanded specimen base now available, and to see whether there is recognizable geographic variation. If a modern researcher is uncomfortable with the qualitative descriptions and comparisons of an earlier time, some of the comparisons can now be quantified—color and shape for example.

38. I was unable to associate these points with Table 4. Does this belong elsewhere? Or perhaps these were just someone's notes, erroneously left behind.

Evaluating the subspecies

Testing the Taxonomic Validity of ~~Preble's Meadow Jumping Mouse~~ (*Zapus hudsonius preblei*)

Report to the Governor of Wyoming and U.S. Fish & Wildlife Service (Revised)

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Revised 12 March 2004
New in this report:
- Results of discriminant analysis using repeated skull measurements.
- Haplotype tables and phylogeny revised to be more informative

Abstract

We examined three lines of evidence to test the taxonomic validity of *Z.h. preblei*. These included: 1) phylogenetic and population genetic analysis of 176 mitochondrial DNA sequences, 2) morphometric analysis of skull measurements of 80 individuals, and 3) a critical review of the basis of Krutzsch's qualitative description of *Z.h. preblei* as a subspecies. Phylogenetic analysis of mtDNA sequence data revealed that *Z.h. preblei* was not unique relative to *Z.h. campestris*, all *Z.h. preblei* mtDNA haplotypes were found within individuals of *Z.h. campestris*. *Z.h. luteus* is most closely related to *Z.h. pallidus*. Population genetic analysis revealed greater mtDNA variation within rather than among *Z.h. preblei* and *Z.h. campestris*. The lowest mtDNA variation was found within *Z.h. preblei*. Our morphometric analyses (analysis of variance and linear discriminant analysis of repeated skull measurements) refutes the quantitative morphological basis for Krutzsch's description of *Z.h. preblei* as a subspecies. Rather than being smaller in most skull dimensions than *Z.h. campestris*, *Z.h. preblei* was significantly larger for two measurements, smaller for one, and insignificant for 6 others. Discriminating ability with a jackknifed posterior probability of ≥0.95 was poor, with 48% (35 of 72) of the specimens correctly classified to each subspecies. The skull shape and pelage differences noted by Krutzsch have no quantitative basis and must be considered as "unsupported opinion". The lack of genetic, morphological, or published ecological evidence for distinctiveness of *Z.h. preblei* from *Z.h. campestris*, means that these subspecies should be synonymized (considered the same subspecies - *Z.h. campestris*). *Z.h. preblei* does not appear to be sufficiently unique to qualify as a Distinct Population Segment under the Endangered Species Act.

Introduction:

There is some controversy surrounding the taxonomic validity of Preble's meadow jumping mouse (*Zapus hudsonius preblei*) and conservation efforts under the Endangered Species Act (ESA) based on the presumed genetic uniqueness of this subspecies. This controversy is based upon the apparent weakness of the original taxonomic inference (Krutzsch 1954) which was an important component to the listing of *Z.h. preblei* under the ESA. The weakness of the original taxonomic designation includes: limited numbers of specimens used to describe the subspecies (3 adult skulls, 4 adult skins, 7 juvinile skins), qualitative descriptions that would not meet modern standards, and similarity in physical appearance of *Zapus* species and subspecies. The taxonomy of Krutzsch (1954) was not critically questioned by the scientific community or the USFWS until this study was proposed by the Denver Museum of Nature & Science in August 2002 and the results released in December 2003.

Z.h. preblei is one of 12 subspecies of the meadow jumping mouse (*Z. hudsonius*), a species whose range covers approximately half of North America. The range of *Z. hudsonius* extends from the Pacific Coast of Alaska eastward to the Atlantic Coast; from the northern limit of tree growth south into central Colorado, Nebraska, eastern Kansas, Missouri, Tennessee, and northern Georgia (Krutzsch 1954, Whitaker 1972). The range of *Z.h. preblei* is restricted to the base of the Front Range in Colorado and into southeastern Wyoming. The presumed cause of its uniqueness is the retreat of moist riparian habitat across the eastern plains of Colorado that occurred following the opening of the Holocene, approximately 10,000 years ago (Hafner 1981, 1987).

To date, most of the research has focused on distinguishing *Z. hudsonius preblei* from the western jumping mouse (*Z. princeps princeps*). Connor and Shenk (2003) used discriminant analysis of skull measurements to distinguish specimens of *Z. h. preblei* from *Z. princeps princeps*. An unpublished report by Riggs et al. (1997) claimed that based on mitochondrial control region sequences *Z. h. preblei* forms "a homogenous group recognizably distinct from nearby populations and adjacent species of the genus." However, these authors did not gather data in such a manner as to be able to rigorously test whether *preblei* formed a monophyletic group. Furthermore Riggs et al. did not provide any statistical tests to support their conclusions. The data set used in the unpublished report by Riggs et al. (1997) is privately held by by Biosphere Genetics Inc, Berkeley, CA.

If *Z. hudsonius preblei* is found to be indistinguishable from other subspecies of *Z. hudsonius*, then conservation efforts under the Endangered Species Act are being directed toward an organism that is more common and widespread then previously thought. If *Z. h. preblei* is found to be unique, relative to other subspecies of *Z. hudsonius*, then it may deserve conservation attention under the ESA, so long as it does not freely hybridize with *Z. princeps*, a common species whose distribution may overlap the western boundary of *Z. h. preblei*.

We tested the genetic distinctiveness and taxonomic validity of the Preble's meadow jumping mouse relative to other subspecies of the same species that are found in

neighboring
~~bord~~ing states. Our comparisons included samples of *Z. h. luteus* (from New Mexico and Arizona), *Z. h. campestris* (from Wyoming,, Montana, and South Dakota), and *Z. h. pallidus* (from Kansas and Nebraska). We used phylogenetic and population genetic methods to analyze DNA sequence data, as well as modern subspecies and distinct population concepts (Ball and Avise 1992, Crandall et al. 2000). We also ~~retested~~ re-examined Krutsch's original conclusions regarding cranial differences between *Z. h. preblei* and *Z. h. campestris*, using larger sample sizes. And finally, we ~~examined~~ re-analyzed Krutzsch's qualitative descriptions of skull shape and pelage differences between *Z. h. preblei* and *Z. h. campestris*.

1) Analysis of Mitochondrial DNA sequence variation

Methods:

Conceptual approach:

We used the scientific method to provide an objective test of the genetic distinctiveness of the Preble's meadow jumping mouse. Using hypotheses laid out in advance of data collection, we used the criteria of Ball and Avise (1992) and Moritz (1994) to test the taxonomic uniqueness of *Z. h. preblei* relative to other subspecies of *Z. hudsonius*. These authors were the first to provide a conceptual basis for recognizing subspecies (which are generally equated with evolutionary significant units or ESUs) that has both an evolutionary and quantitative basis. Ball and Avise (1992), and Moritz (1994) provided the following criteria for recognizing subspecies or ESU's: the subspecies or ESU must represent a major division in the diversity of the gene pool of a species based on concordant distributions of multiple genetically-based traits; it must have a plausible evolutionary mechanism for differentiation, and it must be on separate mitochondrial DNA lineages (reciprocal monophyly). The criteria of reciprocal monophyly for mitochondrial DNA requires that subspecies be separated long enough (e.g. generations since separation = 2 times the effective population size) for them to be on separate evolutionary pathways. While strict reciprocal monophyly is a clear-cut standard, it may be refuted if additional sampling reveals even one shared mitochondrial DNA type among subspecies. We prefer a less restrictive standard, specifically, there must be greater diversity between putative subspecies than within them. We previously used the approach outlined above in taxonomic revision of wild sheep (Ramey 1995, Wehausen and Ramey 2000, Tserenbatta et al. in press).

In our original research proposal "Testing the Taxonomic Validity of the Preble's Meadow Jumping Mouse", we asked the following question "Are Preble's meadow jumping mice a unique subspecies relative to other nearby *Z. hudsonius* subspecies?" We then laid out the following hypotheses and critical tests:

"Hypothesis 1A: Preble's is a unique taxon, distinguishable from other subspecies of *Z. hudsonius* using mitochondrial DNA sequence data. The alternative hypothesis (Hypothesis1B) is that Preble's ~~will~~ not be unique or distinguishable.

Critical test: Mitochondrial DNA sequence data for all samples show a pattern of reciprocal monophyly, or greater molecular variance among subspecies than within subspecies (in pairwise comparisons involving *Z. h. preblei*.) If we find that Preble's

cannot be distinguished on the basis of mitochondrial DNA sequences, it will be unlikely that it will be differentiated for nuclear microsatellite DNA. However, if Hypothesis 1A cannot be refuted, then screening all samples for microsatellite loci becomes crucial to test if hybridization occurs between *Z. h. preblei* and *Z.p. princeps*."

Following our initial test using the criteria above, we also applied the conceptual approach of Crandall et al. (2000). These authors propose a hypothesis testing approach for recognizing distinct population segments using the criteria of genetic and ecological distinctiveness on recent and historic timescales. They advocate that ecological differences among populations can drive adaptive change that would not be detected by molecular markers alone. Therefore, we examined the literature for evidence of ecological differences between subspecies. We applied the conceptual approach using the crosshair classification of Table 1 in Crandall et al. (2000). We define "recent" as within the past 10,000 years (Holocene) and "very recent" as within the past several hundred years.

Acquisition of samples:
DNA samples were obtained from specimens in museum collections at the Denver Museum of Nature & Science, the University of Kansas, the Nebraska State Museum, and the University of New Mexico. We included only two ear punch tissue samples from live captured animals because they were needed to fill in a sampling area and photographs of these individuals were available . By relying on museum specimens, our results are repeatable. Additional questions may also be asked about each specimen at a later date, such as morphological distinctiveness. Museum research collections have the advantage of being open to public inspection and scientific research.

We sampled across the range of each putative subspecies, in order to sample the maximum extent of genetic variation across subspecies. This meant that we sampled more locations but fewer individuals per location. We included a limited sample from each of the subspecies of *Z. princeps* for use as an outgroup for phylogenetic analyses. Previous work by J. Cook (unpublished data) revealed a broad separation and reciprocal monophyly between *Z. princeps* and *Z. hudsonius* utilizing cytochrome *b* sequences, making *Z. princeps* an ideal outgroup for phylogenetic analyses.

Laboratory Methods:
Genomic DNA was extracted from frozen liver tissue and museum skin samples (5-10mg) using Qiagen DNeasy Tissue kit (Qiagen Inc.). Two specimens were from ear punch samples provided by Pioneer Environmental that had accompanying photographs (virtual vouchers). For frozen tissues, we followed the protocol provided in the Qiagen DNeasy Tissue kit. For skin samples, we modified the protocol slightly – samples were incubated at ATL buffer with proteinase K overnight at 56°C. 510bp of control region were amplified via the polymerase chain reaction (PCR) using primer L15320 and ZAP5P1r. The amplification conditions were as follows: in a 25 µl total volume, containing 5 µl of Invitrogen optimizer buffer D (17.5 mM $MgCl_2$, pH 8.5) (Invitrogen, Inc.), 2.5 µl of dNTPs (2.5 mM each), 1.25 µl of each primer (10 µM), 1 unit *Taq* polymerase, 1µl of template (200-300 ng), and 13.8 µl of sterile water. The temperature

profile for the PCR reaction consisted of an initial 2 min denaturation step at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C, and a final extension step at 72°C for 7 min. Amplified DNA was resolved by electrophoresis on 1.5% agarose gel that was stained with ethidium bromide to check for length, quality and quantity.

Some DNA extracts, most notably those of older museum specimens (prior to 1980), did not amplify well or at all. We suspect that this occurred because the older museum specimens were treated with arsenic during skin preparation. We were able to amplify DNA from these older museum specimens using nested PCR. Two primers, L15398 and H16498 were designed to amplify ca. 430 bp control region fragment within the L15320/ZAP5P1r primer combination. The relative positions and priming directions of the control region primers are shown in Figure 1. Genomic DNA was first amplified using primer L15320 and ZAP5P1r. The PCR products were cleaned using the Exo/SAP method. The PCR products were incubated at 37°C for 30 min and then at 85°C for another 15 min with five units of Exonuclease I (ExoI, Amersham) and 0.5 unit Shrimp Alkaline Phosphatase (SAP, Amersham). Subsequently the cleaned PCR product was reamplified using primer L15398 and H16498.

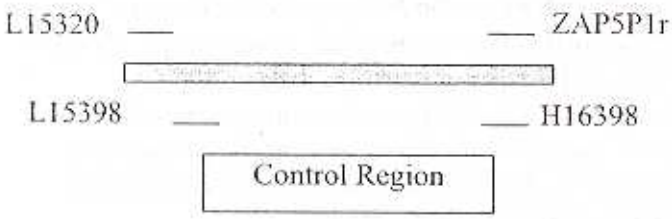

Figure 1. Location of primers used for PCR amplification of mitochondrial Control Region.

Automated Sequencing. The amplified PCR product was incubated at 37°C for 30 min and then at 85°C for another 15 min with five units of Exonuclease I (ExoI, Amersham) and 0.5 unit Shrimp Alkaline Phosphatase (SAP, Amersham) to cleave nucleotides one at a time from an end of excess primers and to inactivate single nucleotides. Approximately 10-30 ng of cleaned PCR product was used as a template in a cycle sequencing reaction using the CEQ DTCS Quick Start Kit (Beckman Coulter, Inc.). The following cycling conditions were used: 96°C for two min, then 30 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for four min. The cycle-sequenced product was cleaned using the Beckman Coulter protocol. Fluorescent dye-labeled DNA was combined with 4 µl stop solution (equal volume of 100 mM EDTA and 3 M NaOAc pH 5.2), 1 µl glycogen (20 mg/ml), and 10 µl milli-Q H_2O, mixed well, and precipitated with 60 µl cold 95% (v/v) ethanol/water. Fluorescent dye-labeled DNA was recovered by centrifuging at 13,000 rpm for 20 min at 4° C. Pellets were washed with 100 µl 70% (v/v) ethanol/water, air dried and resuspended in 40 µl of dimethylformamide. Resuspended samples were added

to the appropriate wells of the CEQ sample plate, overlayed with mineral oil, and run on the Beckman Coulter CEQ8000. Sequences were determined for both strands and were edited and aligned using Sequencher™. All DNA sequences were determined by sequencing in the forward and reverse directions, with additional runs used to eliminate ambiguous base calls. Aligned and edited sequences were checked back against raw chromatograms to insure base calling accuracy.

Data Analysis. Consensus sequences were aligned using Sequencher and verified manually. Phylogenetic hypotheses based on distance and parsimony methods were conducted using PAUP* 4.0b10 (Swofford, 2002). A Bayesian analysis using MrBayes 3.04 (Huelsenbeck and Ronquist, 2001) was conducted as another means of estimating phylogeny. The HKY model with variable sites assumed to follow a discrete gamma distribution (e.g., HKY + I+ Γ; Hasegawa et al., 1985) was selected as the best fit for the dataset (Modeltest 3.06; Posada and Crandall, 1998).

Maximum-parsimony (MP) analyses were conducted with equal weighting, using the heuristic search option with tree bisection reconnection branch-swapping and 10 random additions. Bootstrapping with 1000 replications (as implemented in PAUP*) was used to evaluate node support. HKY distances were used to generate a neighbor-joining (NJ) tree based on the clustering method of Saitou and Nei (1987). Node support was assessed by completion of 1000 bootstrap replications (Felsenstein, 1985) in PAUP*, using the fast-search option. Bayesian analyses were performed based on the HKY model with invariable and variable sites with a discrete gamma distribution (e.g., HKY + I+ Γ; Hasegawa et al., 1985) model of evolution. Several short runs were first conducted using the default random tree option to determine when the log likelihood sum reached a stable value (by plotting the log-likelihood scores of sample points against generation time). Then metropolis-coupled MCMC simulations were run with four chains using the default random tree option for 1,000,000 generations and Markov chains were sampled at intervals of 10 generations to obtain 100,000 sample points. The last 95,000 sampled trees with branch lengths (the first 5000 trees having been removed as "burn-in") were used to generate a 50% majority rule consensus tree. The percentage of samples that recovered specific clades on this topology represents that clade's posterior probability; these are the P values, and P ≥ 95% was considered evidence of significant support for a clade (Huelsenback and Ronquiest, 2001).

ARLEQUIN 2.0 was used to perform an analysis of molecular variance (AMOVA) to partition the amount of genetic variation in a hierarchical fashion within and between the most closely related subspecies to *Z. h. preblei* (Excoffier et al. 1992). Statistical significance of differentiation at these levels was quantified and tested using ARLEQUIN 2.0 (Schneider et al. 2000). ARLEQUIN 2.0 was also used to estimate mtDNA nucleotide diversity.

Results:

We sequenced mitochondrial control region from 58 *Z. hudsonius preblei*, 33 *Z. h. campestris*, 32 *Z. h. luteus*, 35 *Z. h. pallidus*, 7 *Z. princeps princeps*, 3 *Z. p. idahoensis*,

and 7 *Z. p. utahensis*. The alignment of 151 sequences (Table 1), excluding four specimens from Wyoming, one from Kansas, one from Montana, and one from South Dakota (see explanation below), of the partial mitochondrial control region from four *Zapus hudsonius* subspecies yielded 355 bp. Overall nucleotide composition was biased towards thymine (T)(34.3%) and adenine (A)(29.8%), followed by cytosine (C)(26.0%) and guanine (G)(9.9%).

Three variable sites (all transitions) were observed among 54 specimens of *Z. h. preblei* resulting in four haplotypes. [Note: four specimens of *Z. h. preblei* from Albany Co., Wyoming had almost identical sequences to *Z. p. princeps*. These four specimens were presumed misidentified and thus not included.] Twenty-nine variable sites (19 transitions, 8 transversions, and 2 indels) were observed among 31 specimens of *Z. h. campestris* resulting in sixteen haplotypes. Four sequences (two haplotypes) of *Z. h. campestris*, three from Lawrence Co., South Dakota and one from Crook Co., Wyoming, are more similar to sequences of *Z. h. luteus* and *Z. h. pallidus* than to other sequences of *Z. h. campestris*. One specimen of *Z. h. campestris* from Carter Co., Montana, and one specimen from Custer Co., South Dakota has similar sequences to *Z. p. utahensis*. We presume they were misidentified and thus not included (Table 2).

Thirty variable sites were observed among 34 specimens of *Z. hudsonius pallidus* resulting in twelve haplotypes. Two sequences of *Z. h. pallidus* from Clay Co., South Dakota are more similar to sequences of *Z. h. campestris* and *Z. h. preblei* than to other sequences of *Z. h. pallidus*. One specimen of Z. h. pallidus from Douglas Co., Kansas, has similar sequences to *Z. p. utahensis*. They are presumed misidentified and thus not included. Six variable sites were observed among 32 specimens of *Z. h. luteus* resulting in eight haplotypes.

Phylogenetic analysis of mtDNA sequences based on maximum parsimony, distance and Bayesian methods yielded concordant results that differed only in the positioning of terminal taxa (Figure 2, Table 1). Phylogenetic analysis of mtDNA sequence data revealed that *Z. h. campestris* is most closely related to *Z. h. preblei* and that *Z. h. luteus* is most closely related to *Z. h. pallidus*. These two clades had strong bootstrap support (Figure 2). *Z. h. preblei* and *Z.h. campestris* were not reciprocally monophyletic. All four of the mtDNA haplotypes found in *Z. h. preblei* were also found in *Z. h. campestris*. No unique mtDNA haplotypes were found in *Z. h. preblei*.

Genetic variation within subspecies as indicated by mtDNA nucleotide diversity was lowest in *Z. h. preblei* (0.0027, SD=0.0020) and approximately nine times higher in *Z. h. campestris* (0.0243, SD=0.0129). Nucleotide diversity in *Z.h. luteus* (0.0041, SD=0.0029) was twice that of *Z.h. preblei* but three times lower than in *Z. h. pallidus* (0.0135, SD=0.0075).

In a pairwise comparison between *Z. h. preblei* and *Z.h. campestris*, analysis of molecular variance revealed that most of the genetic variation was within (64%) rather than among these subspecies (37%), thus refuting hypothesis 1A and failing our test of genetic uniqueness. We did not include the highly divergent sequences of the 4 Albany Co.

specimens in this analysis because it is likely that they are specimens of *Z.p. princeps* that were misidentified as *Z. h. preblei* .

Utilizing the criteria of genetic and ecological exchangeability as proposed by Crandall et al. (2000) for distinct populations, the mtDNA data does not refute the hypothesis of historic or recent genetic exchangeability (interbreeding) between *Z.h. preblei* with *Z.h. campestris*. This is because all four *Z.h. preblei* mtDNA haplotypes are found in *Z.h. campestris* from near the Black Hills of South Dakota. These mtDNA haplotypes that are shared between *Z.h. preblei* and *Z.h. campestris* span a range of up to 700km, from central Colorado to southeastern Montana. The fact all *Z.h. campestris* haplotypes are not found in the range of *Z.h. preblei* is consistent with founder effects and range expansion, not evidence of restricted genetic exchange. A review of the literature reveals that no quantitative evidence exists to reject the hypotheses of historic or recent ecological exchangeability (ecological similarity) between *Z.h. preblei* with *Z.h. campestris*. While it is possible that genetic exchange between these two putative subspecies is currently limited, this alone does not support them as being recognized as a distinct population segment (case 8, Crandall et al. 2000).

Discussion:
Our analysis of mtDNA sequence data refutes Hypothesis 1A, that *Z.h. preblei* is a unique taxon, distinguishable from other subspecies of *Z. hudsonius* (in this case *Z.h. campestris*) using mitochondrial DNA sequence data. The results of the mtDNA analysis reveal that *Z.h. preblei* is a less genetically variable population of *Z.h. campestris*.

The high level of mtDNA variation (nucleotide diversity) found in *Z.h. campestris* compared to *Z.h. preblei* does inflate the F_{ST} estimate, making these subspecies seem more diverged than the shared mtDNA haplotypes indicate.

While it is possible that the low level of mtDNA variation found in *Z.h. preblei* is the result of isolation and a northern migration into the range of *Z.h. campestris*, the pattern is more consistent with the hypothesis that the range of *Z.h. preblei* is the result of a recent southward colonization from the range of *Z.h. campestris*. Two observations support this latter conclusion: first, no unique mtDNA haplotypes were found in *Z.h. preblei* and second, all of these haplotypes were closely related. The reduced mtDNA variation is consistent with a founder effect (e.g. population bottlenecks during a southern colonization). In contrast, if *Z.h. preblei* had been a long term resident along the Front Range and had evolved in isolation from *Z.h. campestris*, more unique mtDNA haplotypes would be expected – a situation found with *Z.h. luteus* compared to *Z.h. pallidus*. In either case, the shared mtDNA haplotypes indicate recent genetic exchange.

The failure of evidence to reject hypotheses of genetic and ecological exchangeability between *Z.h. preblei* with *Z.h. campestris*, using the approach of Crandall et al. (2000), means that *Z.h. preblei* with *Z.h. campestris* should be treated as a single population. If evidence from future trapping efforts supports a lack of current genetic exchangeability (e.g. genetic isolation) between *Z.h. preblei* and *Z.h. campestris*, these two subspecies

would still be considered a single population for management purposes, using the criteria proposed by Crandall et al. (2000).

2) Morphometric analyses: Retesting Krutzsch's conclusions with larger sample sizes, analysis of variance, and discriminant analysis.

Methods:

To test the hypothesis that size differences in skull measurements reported by Krutzsch (1954) are representative of differences among subspecies, we compared 39 adult *Z.h. preblei* and 41 adult *Z.h. campestris* specimens using analysis of variance (ANOVA). Specimens were measured at the zoology collections at the Denver Museum of Nature & Science, and the University of Kansas Museum of Natural History. We utilized the same 9 skull measurements of Krutzsch (1954): occipitonasal length (from anteriormost projection of nasal bones to posteriormost projection of supraoccipital bone), condylobasal length (posteriormost part of exoccipital condyles to anteriormost projections of premaxillary bones), palatal length (anterior border of incisors to anteriormost point of postpalatal notch), zygomatic length (anteriormost point of zygomatic process of maxillary to posteriormost point of zygomatic process of squamosal), zygomatic breadth (greatest distance across zygomatic arches ~~of cranium~~ at right angles to long axis of skull), mastoidal breadth (greatest distance across mastoid bones perpindicular to long axis of skull), braincase breadth (greatest distance across braincase perpindicular to long axis of skull), interorbital breadth (least distance across top of skull between orbits), and upper tooth row length (anterior border of P4 to posterior border of M3). Our palatal length is larger than what Conner and Shenk (2003) reported due to differences in where measurements were taken.

Four repeated measurements (Conner and Shenk 2003) were taken with digital calipers and recorded to the nearest hundredth of a millimeter. Only adult skulls were measured, as determined by tooth eruption and wear. In a several cases, fewer measurements were taken because of breakage or not taken because of previous breakage. Calipers were moved away from the skull and reset for each measurement. A single observer (L. Carpenter) measured all skulls in the study. We used the mean of the repeated measurements in both ANOVA and discriminant analysis (Connor and Shenk 2003).

We tested the cranial distinguishability of *Z.h. preblei* from *Z.h. campestris* from a multivariate perspective with linear discriminant analysis using SYSTAT 9.0. Forward, backward, and interactive stepwise procedures to develop the simplest discriminant models to eliminate statistically unimportant variables and to maximize the ratio of sample size to variables included in the model (Williams and Titua 1990). We used jackknifed estimates of posterior probabilities and classification ability for discriminant models (Afifi and Clark 1990). We used a previously published criterion for testing the hypothesis of distinguishability between subspecies: ≥90% of specimens correctly classified at jackknifed posterior probabilities of $p \geq 0.95$ (Wehausen and Ramey 2000). This criterion was more discriminating than just the percentage of specimens correctly classified at a posterior probability of $p > 0.5$. Males and females were pooled in the analyses because of a lack of cranial sexual dimorphism in *Z. princeps* and *Z. hudsonious*

(Connor and Shenk 2003). This apparent lack of sexual dimorphism was also tested using stepwise discriminant analysis.

Results:

Analysis of variance

Our analysis of skull measurement data refutes the hypothesis above and the claim made by Krutzsch (1954) that *Z.h. preblei* is *"averaging smaller in most skull measurements"* than *Z.h. campestris*. A total of 3 measurement variables were found to be significantly different at a level of $p<0.05$. Two of these measurements (zygomatic breadth and mastoid breadth, were significantly ***larger*** in *Z.h. preblei* than in *Z.h. campestris*, in the *opposite* direction to Krutzsch's claims that *Z.h. campestris* is larger. *Z.h. campestris* was only larger for one measurement (*interorbital breadth*) and it was only marginally significant (larger in *Z.h. campestris)* ($p=0.037$). All other measurements were not significantly different (Table 3).

Discriminant analysis

Four variables were determined to have the greatest discriminating power. These included: zygomatic breadth, mastoidal breadth, breadth of skull, and condylobasal length. A total of 33 *Z.h. preblei* and 39 *Z.h. campestris* were used in the discriminant analysis. The null hypothesis of equal covariances among subspecies was not rejected ($p=0.147$). Discriminating ability with a jackknifed posterior probability of ≥ 0.95 was poor, with 48% (35 of 72) of the specimens correctly classified to each subspecies.

Discussion:

Our morphometric analysis refutes the quantitative morphological basis for Krutzsch's description of *Z.h. preblei* as a subspecies. Krutzsch (1954) described *Z.h. preblei* as *"averaging smaller in most skull measurements"* but using ANOVA, we found only one out of nine variables to be significantly smaller in *Z.h. preblei*. The three significant differences that we did find should be viewed within the context of variation typically found among populations.

Z.h. preblei failed the test of morphological distinguishability from *Z.h. campestris* using discriminant analysis of the same skull measurements as Krutzsch (1954) and a substantially larger sample size. The correct classification of specimens by the DFA was far less (48%) than the criterion that ≥90% of specimens be correctly classified at jackknifed posterior probabilities of $p\geq 0.95$ (Wehausen and Ramey 2000). This is a refutation of Krutzsch's (1954) only quantitative basis for concluding that *Z.h. preblei* are morphologically distinguishable and therefore a unique subspecies relative to *Z.h. campestris*.

As with other taxonomy papers of the period, Krutzsch's description in 1954 of *Z. h. preblei* as a newly recognized subspecies was based upon qualitative descriptions without statistical tests, and presumed geographic isolation. ~~It represented the opinion of the author.~~ The only quantitative comparison that Krutzsch (1954) used to support this "new" subspecies description, was based on measurements of only 3 adult specimens of

Preble's that he compared to 40 specimens of *Z.h. campenstris*. He examined the skin of a fourth adult specimen and the skins of 11 juveniles of *Z. h. preblei*. The three adult *Z. h. preblei* specimens were reported to be smaller in all skull dimensions.

3) A critical evaluation of Krutzsch's qualitative descriptions

We examined the basis of Krutzsch's qualitative differences in skull shape and pelage to determine the strength of the evidence that he used to infer that *Z.h. preblei* is a unique subspecies.

Three of the skull shape differences distinguishing *Z.h. preblei* and *Z.h. campestris* noted by Krutzsch (1954) had no reported measurements. Therefore the skull shape differences noted by Krutzsch have no quantitative basis and must be considered as "unsupported opinion". These shape descriptions include: "*incisive foramia not truncate posteriorly; auditory bullae smaller, less well inflated; and frontal region usually more inflated*". Additionally, one of the skull shape differences ("*frontal region usually more inflated*") did not have an accompanying qualitative description for either subspecies individually (Table 4).

When Krutzsch's pelage descriptions of each subspecies are listed side by side (Table 2), and compared to what he stated were distinguishing pelage differences, it is clear that two of the three pelage differences were made without a description of one or both subspecies. For example, one pelage difference ("*upper parts generally dull, averaging lighter*") had no comparative description for *Z.h. campestris*. The second pelage difference ("*sides duller*") did not have an accompanying description for either subspecies. The only pelage difference where there was a description for both subspecies was "*less black tipped hair*" on the dorsal band. These three differences in pelage between *Z.h. preblei* and *Z.h. campestris* noted by Krutzsch (1954) are entirely qualitative and must also be considered as "unsupported opinion". The underpinnings of Krutsch's qualitative descriptions are without a quantitative basis, and fail the tests of falsifiability, comprehensiveness, repeatability, and sufficiency required by evidential reasoning (Lett 1990).

Conclusions:

Taxonomy

We examined three lines of evidence to test the taxonomic validity of *Z.h. preblei*. These included: 1) phylogenetic and population genetic analysis of mitochondrial DNA sequences, 2) morphometric analysis of skull measurements, and 3) a critical review of the logical basis of Krutzsch's description of *Z.h. preblei* as a subspecies. Our results failed to support the genetic distinctiveness of *Z.h. preblei* from *Z.h. campestris*. Our morphometric analysis refutes the quantitative morphological basis for Krutzsch's description of *Z.h. preblei* as a subspecies. The skull shape and pelage differences noted by Krutzsch have no quantitative basis and must be considered as "unsupported opinion".

The lack of genetic, morphological, or published ecological evidence for genetic distinctiveness (including adaptive divergence) of *Z.h. preblei* from *Z.h. campestris*, means that these subspecies should be synonymized (considered the same) and referred to as *Z.h. campestris*.

The lack of genetic, morphological, and ecological evidence supporting divergence of *Z.h. preblei* from *Z.h. campestris*, the weakness of the original taxonomic inference of *Z.h. preblei* being a subspecies (Krutzsch 1954), and the unsupported assumption that geographic isolation has driven genetic divergence between these putative subspecies, all point to *Z.h. preblei* being synonymous with *Z.h. campestris*. We therefore synonymize *Z.h. preblei* with *Z.h. campestris*

Does the evidence support consideration of Distinct Population Segment listing?

In a broader perspective, the range of *Z.h. preblei* represents less than 5% of the range of a species whose range is approximately *half* of North America (along streams and in meadows). This is not a compelling argument for *Z.h. preblei* to be a candidate for a distinct population segment designation (DPS). A DPS designation requires that a population be "discrete" and "of significance" (US Fish & Wildlife Service 1996). The "discrete" requirement, that a DPS is "markedly separated from other populations of the same taxon by physical, physiological, ecological, or behavioral factors" using evidence from "quantitative measures of genetic or morphological discontinuity" (US Fish & Wildlife Service 1996) is not supported by our genetic or morphological analyses.

The "significance" requirement that, "evidence that loss of the discrete population segment would result in a significant gap in the range of a taxon" is not supported because of the broad distribution of *Z. hudsonius* (Figure 3). *Z.h. preblei* is a peripheral population of *Z. hudsonius* that does not rank as distinct using the criteria (spatial distance, life history, time, and ecology) proposed by Lesica and Allendorf (1994).

Hypothesis testing and peer review

Krutzsch's (1954) unsupported opinions about shape differences in skulls and coloration of skins, as well as skull measurement comparison based on a sample size of 3 *Z.h. preblei*, have carried the weight as the "best available science" in the listing of *Z.h. preblei*. However, the logical basis of these opinions was not critically evaluated by the USFWS, or others, during the listing process, despite the weakness of Krutzsch's (1954) inference by modern standards. The identification of *Z.h. preblei* specimens by museum curators or consultants similarly relied on Krutzsch (1954). The description of *Z.h. preblei* as a new subspecies is typical of the taxonomic work that appeared in the literature in the early to mid twentieth century. During that time, species and subspecies descriptions had little or no quantitative basis, relied on small sample sizes, and were based largely on opinion (Ramey 1993, Wehausen and Ramey 1993, 2000). Essentially, a species or subspecies was "*what a good taxonomist said it was*".

The original review of the *Z.h. preblei* listing would have benefited from a critical peer review by more broadly trained systematic biologists and molecular/morphometric analyses to specifically test the taxonomic validity of subspecies. The Federal peer review standards proposed by the Office of Management and Budget (2003) are a good example of how peer review can strengthen the scientific justification for proposed ESA listings, delistings, and Biological Opinions. Also, genetic analyses with the specific goal of treating taxonomic categories as testable hypotheses (Ramey 1993, 1995; Wehausen and Ramey 1993, 2000) would have been appropriate in this case and others. In the case of *Z.h. preblei*, a genetic analysis was performed by Riggs et al. (1997) but not with the subspecies validity question in mind or critical hypothesis testing. Similarly, the listing rule (USFWS 1998) appeared to have accepted the taxonomy of Krutzsch (1954) without question. Our review differs from those previously (Riggs et al. 1997; Hafner 1997; USFWS 1998) because it involves hypothesis testing, utilizes multiple lines of evidence, and incorporates modern concepts of subspecies and distinct population segments. Our analyses suggest that a large expenditure of conservation effort under the ESA is being directed towards populations of a subspecies (*Z. h. campestris*) that are more widespread than previously thought.

Scientific investigation involves critical thinking and evidential reasoning (Lipps 1999, Lett 1990, Platt 1964). Unsupported opinion and anecdotal observations are not scientific. In the case of endangered species management, facts (quantitative evidence) can be gathered in such a way as to answer specific questions, often at greater economy than courses of action whose basis is falsified later. Testing taxonomic classifications does not take as long, or cost as much, as one might initially think. The molecular data has taken approximately one year of part-time effort at a cost of approximately $50,000. Our morphometric measurements, analysis, and write up has taken only three weeks of effort, at a cost of approximately $7,000. Our analyses have benefited greatly from the availability of museum specimens in zoological research collections. Without these collections, this biodiversity research would not have been possible.

In the future, we strongly urge the USFWS to work with the scientific community in developing incentives to apply both critical peer review and molecular/morphometric analyses to test the quantitative basis of all proposed subspecies and distinct population segment listings. To not do so invites a potential for misallocation of scarce conservation resources to populations that are not genetically or ecologically unique, and can erode public confidence in the implementation of the Endangered Species Act.

Acknowledgements:
We thank the State of Wyoming, the U.S. Fish & Wildlife Service, and the Denver Museum of Nature & Science for supporting this research. We thank the curators and collection managers at the Denver Museum of Nature & Science, University of Kansas Natural History Museum, University of New Mexico Museum of Southwestern Biology, and University of Nebraska State Museum, who provided access or loans from zoological and/or genomic research collections. We thank Pioneer Environmental Services and the City of Fort Collins for providing access to the two ear punch specimens. We thank Dr. Tom Quinn and Dr. Sarah Oyler-McCance who provided access to the DNA sequencing

facility at Rocky Mountain Center for Conservation Genetics and Systematics at the University of Denver. Special thanks to Dr. Cheri Jones who expanded and curated the *Z.h. preblei* specimens at DMNS. We are grateful for discussions and constructive criticism throughout this project from the following people: Dr. David Armstrong, Dr. Laura M. Brown, Dr. Norm Clippinger, Dr. Joseph Cook, Clint Epps, Kristin Hintz, Dr. Cheri Jones, Dr. Carron Meany, Dr. Jeff Mitton, Bruce Roselund, Dr. Vern Stelter, and Dr. John Wehausen.

Comments and constructive criticisms on this report are appreciated. Please direct these to rramey@dmns.org. Thank you.

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Appendix I:

Catalog of specimens examined for skull morphometry. Specimens are listed in the order they were examined.

Denver Museum of Nature & Science, *Z.h. preblei*: 9572, 9864, 10380, 9843, 9853, 9570, 9569, 9562, 9561, 9315, 9205, 9204, 9868, 9862, 10355, 10404, 10269, 10354, 10169, 10265, 10267, 2822, 10604, 9876, 10618, 10630, 10621, 9564, 9312, 10635, 9877, 10620, 10611, 9571, 10266, 10610, 9579, 10613, and 10615. Denver Museum of Nature & Science, *Z.h.campestris*:8512. University of Kansas Natural History Museum, *Z.h.campestris*: 101551, 101552, 101554, 101555, 101558, 101560, 87040, 87041, 87042, 87034, 87035, 87036, 87037, 112664, 112657, 20835, 20836, 20837, 20838, 20839, 20840, 20842, 20843, 20844, 20845, 20846, 20847, 20848, 20849, 20851, 20850, 20852, 41450, 41451, 42467, 42468, 42469, 42471, 42517, and 42518.

Figure 2. Neighbor-joining phylogram based on partial control region sequences using a HKY substitution model, depicting phylogenetic relationships among subspecies of *Zapus hudsonius*. One hundred seventy six sequences were obtained for this study (Table 1 and 2). In order to provide a reasonable size tree, one sequence from each haplotype was used. Bootstrap percentages are given when >=50%. Other methods of phylogenetic analysis produced very similar trees.

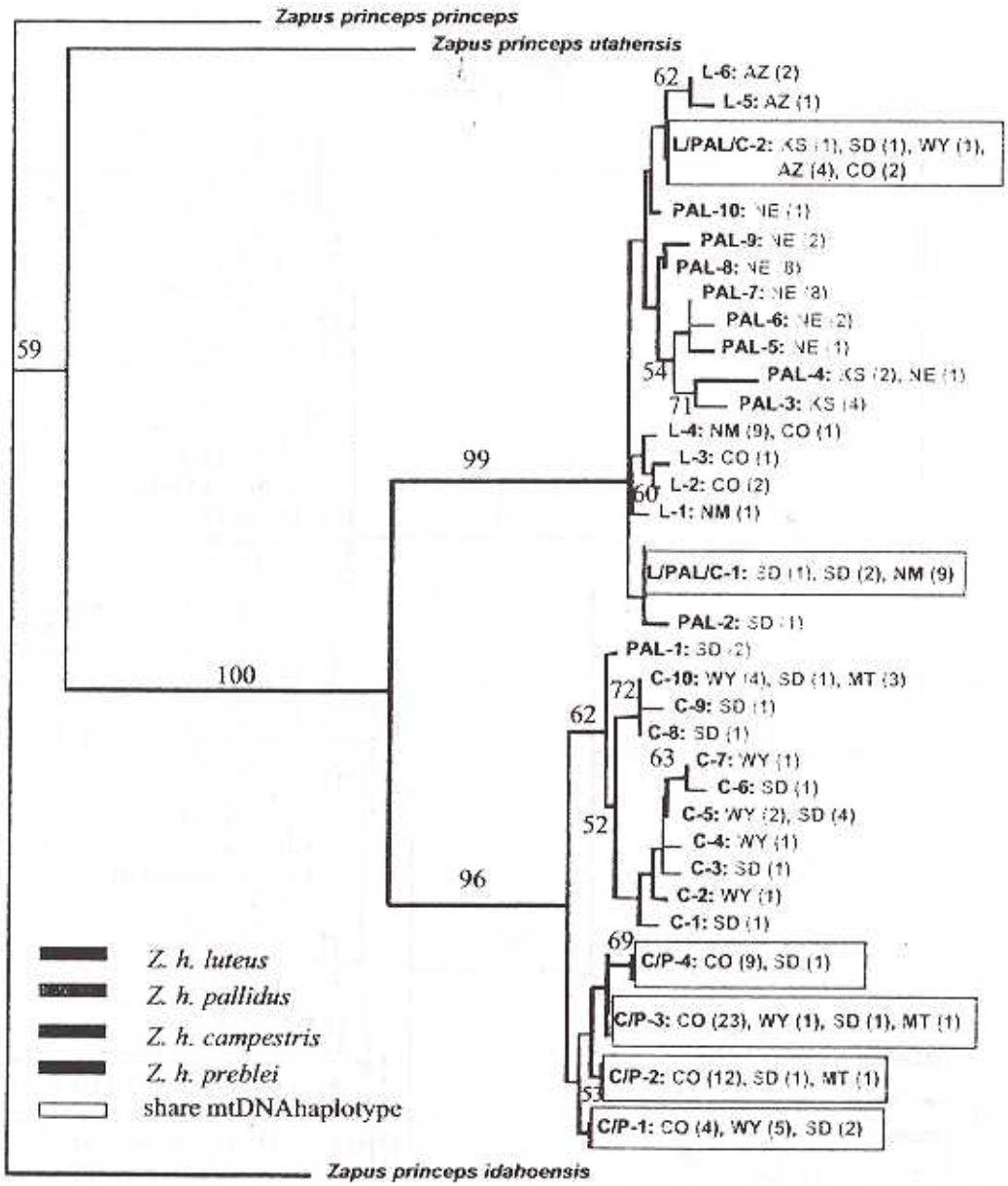

Figure 2. Neighbor-joining phylogram based on partial control region sequences using a HKY substitution model, depicting phylogenetic relationships among subspecies of *Zapus hudsonius*. One hundred seventy six sequences were obtained for this study (Table 1 and 2). In order to provide a reasonable size tree, one sequence from each haplotype was used. Bootstrap percentages are given when >=50%. Other methods of phylogenetic analysis produced very similar trees.

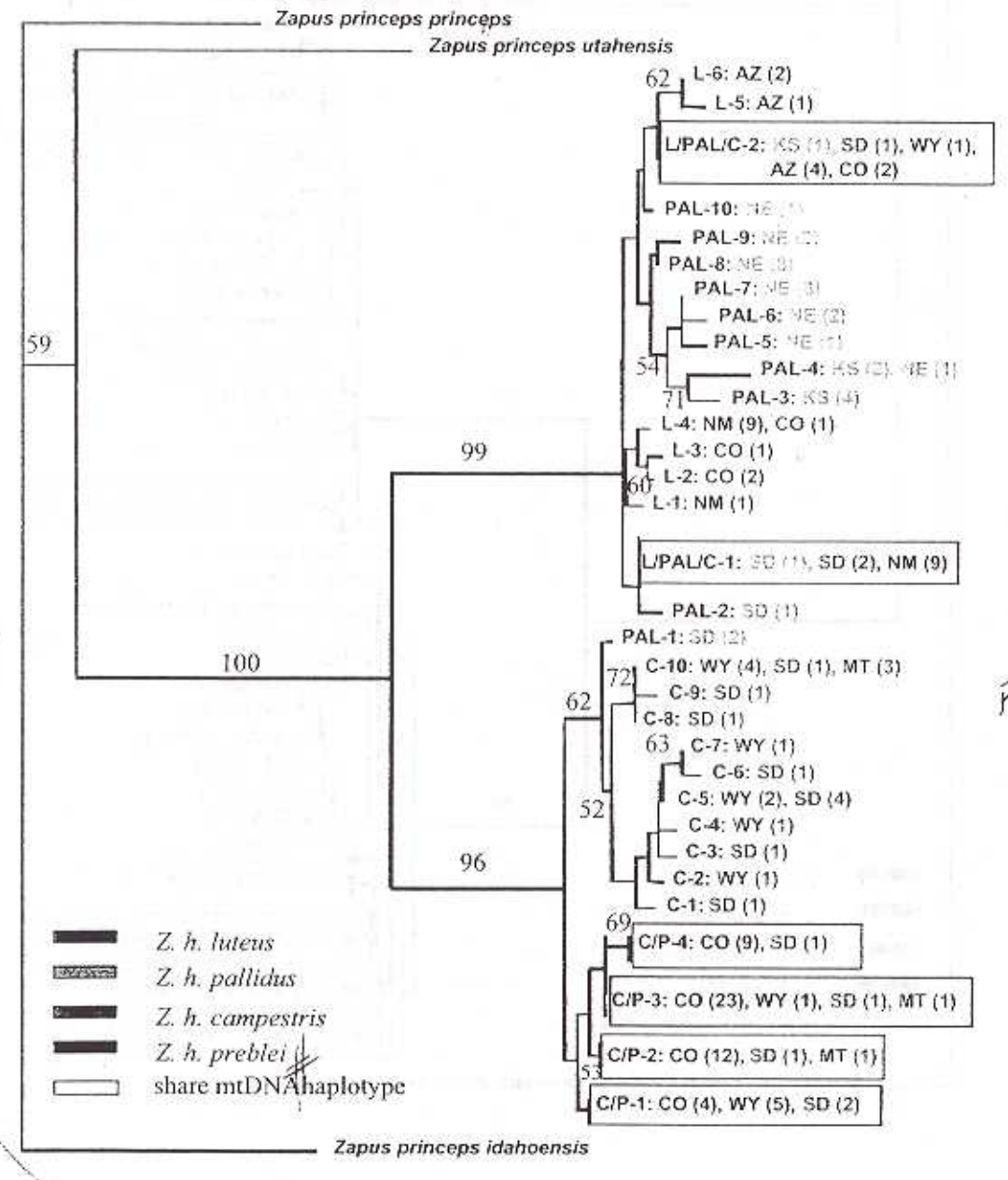

Table 1. Specimens of *Z. hudsonius* used in phylogenetic analysis, listed by museum and tissue archive catalog number (DMNH = Denver Museum of Nature & Science; TK = Texas Tech (tissue archive); KU = University of Kansas; UNSM = University of Nebraska State Museum; MSB and NK (Tissue archive) = Museum of Southwestern Biology; PIONEER = Pioneer Environmental Services.)

Representative individuals used in phylogenetic analysis	Additional specimens with identical mtDNA haplotype:.ID, state, and county	subspecies	haplotype
MSB40951, AZ:Apache		Z.h. luteus	L6
	MSB40994, AZ:Apache	Z.h. luteus	
MSB89194, AZ:Navajo		Z.h. luteus	L5
MSB86344, AZ:Apache		Z.h. luteus	L/PAL/C2
	MSB91627, AZ:Navajo	Z.h. luteus	
	MSB91675, AZ:Apache	Z.h. luteus	
	NK1584, AZ:Apache	Z.h. luteus	
	DMNH8635, CO:Las Animas	Z.h. luteus	
	DMNH8633, CO:Las Animas	Z.h. luteus	
	KU41451, WY:Crook	Z.h. campestris	
	KU153706, KS:Leavenworth	Z.h. pallidus	
	KU112661, SD: Lawrence	Z.h. campestris	
UNSM20596, NE:Buffalo		Z.h. pallidus	PAL10
UNSM26492, NE:Buffalo		Z.h. pallidus	PAL9
	UNSM20879, NE:Buffalo	Z.h. pallidus	
UNSM13217, NE:Cherry		Z.h. pallidus	PAL8
	UNSM12980, NE:Garden	Z.h. pallidus	
	UNSM12991, NE:Garden	Z.h. pallidus	
	UNSM26316, NE:Hall	Z.h. pallidus	
	UNSM20744, NE:Hall	Z.h. pallidus	
	UNSM20747, NE:Hall	Z.h. pallidus	
	UNSM26462, NE:Merrick	Z.h. pallidus	
	UNSM13067, NE:Thomas	Z.h. pallidus	
UNSM17482, NE:Antelope		Z.h. pallidus	PAL7
	UNSM17495, NE:Antelope	Z.h. pallidus	
	UNSM17498, NE:Antelope	Z.h. pallidus	
	UNSM17499, NE:Antelope	Z.h. pallidus	
	UNSM13084, NE:Dixon	Z.h. pallidus	
	UNSM14008, NE:Dodge	Z.h. pallidus	
	UNSM13118, NE:Holt	Z.h. pallidus	
	UNSM13343, NE:Lancaster	Z.h. pallidus	
UNSM13119, NE:Holt		Z.h. pallidus	PAL6
	UNSM13065, NE:Thomas	Z.h. pallidus	
UNSM17727, NE:Boyd		Z.h. pallidus	PAL5
UNSM20600, NE:Buffalo		Z.h. pallidus	PAL4
	KU109633, KS:Osage	Z.h. pallidus	
	KU109634, KS:Osage	Z.h. pallidus	

KU153597, KS:Macon	KU153598, KS:Macon KU153784, KS:Douglas KU153707, KS:Leavenworth	Z.h. pallidus Z.h. pallidus Z.h. pallidus Z.h. pallidus	PAL3
MSB37154, NM:Otero	MSB61696, NM:Otero MSB61684, NM:Otero MSB61690, NM:Otero MSB61693, NM:Otero MSB61712, NM:Otero MSB58369, NM:Rio Arriba NK871, NM:Otero NK884, NM: Socorro DMNH8630: CO:Las Animas	Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus	L4
DMNH8631, CO:Las Animas		Z.h. luteus	L3
DMNH8632, CO:Las Animas	DMNH8634. CO:Las Animas	Z.h. luteus Z.h. luteus	L2
NK9976, NM:Bernalillo		Z.h. luteus	L1
MSB58370, NM:Rio Arriba	MSB56980, NM:Sandoval MSB56986, NM:Sandoval MSB56987, NM:Sandoval MSB56991, NM:Sandoval MSB56993, NM:Sandoval MSB62096, NM:Sandoval MSB62103, NM:Valencia NK856, NM:Sandavol KU112665, SD:Lawrence KU109963, SD:Lawrence KU110033, SD:Bennett	Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. luteus Z.h. campestris Z.h. campestris Z.h. pallidus	L/PAL/C1
KU110022, SD:Bennett		Z.h. pallidus	PAL2
UNSM27388, SD:Clay	UNSM27389, SD:Clay	Z.h. pallidus Z.h. pallidus	PAL1
DMNH10638/TK86190, WY:Weston	DMNH10639/TK86191, WY:Weston KU101558, SD:Pennington KU123593, MT:Carter KU123598, MT:Carter KU123599, MT:Carter	Z.h. campestris Z.h. campestris Z.h. campestris Z.h. campestris Z.h. campestris Z.h. campestris	C10
KU112663, SD:Lawrence		Z.h. campestris	C9
KU101564, SD:Pennington		Z.h. campestris	C8
KU20839, WY:Crook		Z.h. campestris	C7
KU83559, SD:Harding		Z.h. campestris	C6
KU20844, WY:Crook		Z.h. campestris	C5

	KU42471, WY:Weston	Z.h. campestris	
	KU87040, SD:Harding	Z.h. campestris	
	KU83557, SD:Harding	Z.h. campestris	
	KU87042, SD:Harding	Z.h. campestris	
	KU112660, SD:Lawrence	Z.h. campestris	
KU20843, WY:Crook		Z.h. campestris	C4
KU109970, SD:Lawrence		Z.h. campestris	C3
KU42469, WY:Weston		Z.h. campestris	C2
KU101552, SD:Pennington		Z.h. campestris	C1
DMNH10614/TK86183, CO:El Paso		Z.h. prebleii	C/P4
	DMNH10331/TK86088, CO:Teller	Z.h. prebleii	
	DMNH10606/TK86165, CO:El Paso	Z.h. prebleii	
	DMNH10604/TK86169, CO:El Paso	Z.h. prebleii	
	DMNH10612/TK86170, CO:El Paso	Z.h. prebleii	
	DMNH10605/TK86173, CO:El Paso	Z.h. prebleii	
	DMNH10618/TK86182, CO:El Paso	Z.h. prebleii	
	DMNH10611/TK86185, CO:El Paso	Z.h. prebleii	
	DMNH10635/TK86196, CO:Douglas	Z.h. prebleii	
	KU109972, SD:Custer	Z.h. campestris	
DMNH9204/XM871, CO:Boulder		Z.h. prebleii	C/P3
	DMNH9205/XM872, CO:Boulder	Z.h. prebleii	
	DMNH9312/XM874, CO:Gilpin	Z.h. prebleii	
	DMNH9046/XM876, CO:Boulder	Z.h. prebleii	
	DMNH9314/XM877, CO:Boulder	Z.h. prebleii	
	DMNH9203/TK51406, CO:Jefferson	Z.h. prebleii	
	DMNH9880/TK86021, CO:Boulder	Z.h. prebleii	
	DMNH9854/TK86026, CO:Douglas	Z.h. prebleii	
	DMNH9876/TK86029, CO:Douglas	Z.h. prebleii	
	DMNH9857/TK86030, CO:Douglas	Z.h. prebleii	
	DMNH9865/TK86031, CO:Douglas	Z.h. prebleii	
	DMNH9868/TK86032, CO:Douglas	Z.h. prebleii	
	DMNH9843/TK86034, CO:Boulder	Z.h. prebleii	
	DMNH10169/TK86048, CO:Boulder	Z.h. prebleii	
	DMNH10266/TK86080, CO:Douglas	Z.h. prebleii	
	DMNH10269/TK86083, CO:Douglas	Z.h. prebleii	
	DMNH10354/TK86090, CO:Boulder	Z.h. prebleii	
	DMNH10408/TK86098, WY:Albany	Z.h. prebleii	
	DMNH9564/TK86105, CO:Boulder	Z.h. prebleii	
	DMNH9561/TK86109, CO:Larimer	Z.h. prebleii	
	DMNH9576/TK86115, CO:Douglas	Z.h. prebleii	
	DMNH9574/TK86116, CO:Douglas	Z.h. prebleii	
	DMNH10520/TK86124, CO:Jefferson	Z.h. prebleii	
	DMNH10602/TK86163, CO:Elbert	Z.h. prebleii	
	KU110013, SD:Custer	Z.h. campestris	

	KU123597, MT:Carter	Z.h. campestris	
DMNH9579/XM1166, CO:El Paso	DMNH9313/XM875, CO:El Paso DMNH9315/XM879, CO:El Paso DMNH10380/TK86093, CO:El Paso DMNH9565/TK86106, CO:El Paso DMNH9563/TK86107, CO:El Paso DMNH9566/TK86118, CO:El Paso DMNH9573/TK86120, CO:Douglas DMNH9572/TK86121, CO:Douglas DMNH9571/TK86122, CO:Douglas DMNH9574/TK86166, CO:El Paso DMNH10607/TK86167, CO:El Paso KU109978, SD:Custer KU123592, MT:Carter	Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. campestris Z.h. campestris	C/P2
DMNH10405/TK86095, WY:Albany	DMNH10258/TK86074, WY:Laramie DMNH10270/TK86081, CO:Larimer DMNH10404/TK86094, WY:Platte DMNH10406/TK86096, WY:Albany DMNH10407/TK86097, WY:Albany DMNH9568/TK86117, CO:Larimer PIONEER9A43, CO: Larimer PIONEER9B89, CO:Larimer KU109984, SD:Custer KU109985, SD:Custer	Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. prebleii Z.h. campestris Z.h. campestris	C/P1

Table 2. Specimens of *Z. princeps* used as outgroups in phylogenetic analysis and specimens that have an identical mtDNA haplotype or are on the same clade as the mtDNA haplotypes of representative individuals. Only the mtDNA haplotypes of the three represenative *Z. princeps* individuals were used in phylogenetic analysis. Note that some individuals previously identified as *Z. hudsonicus* have mtDNA haplotypes identical to *Z. princeps*. These individuals were presumed to be misidentified and not included in phylogenetic or population genetic analyses.

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

*Sister taxa on the same clade as resprentative individual, with strong bootstrap support.
For computation & simplicity, these individuals were not used in phylogenetic analysis.

Table 3 Summary statistics for mean of repeated cranial measurements for *Z.h. campestris* and *Z.h. preblei*. Using ANOVA, 3 of the cranial measurements were significantly different ($p<0.05$) between subspecies: zygomatic breadth ($P=0.0071$), mastaoidal breadth ($P=0.012$), and interorbital breadth ($p=0.032$). *Z.h. preblei* was larger for both zygomatic breadth and mastaoidal breadth, while *Z.h. campestris* was larger for interorbital breadth. Using single measurements from three adult specimens of *Z.h. preblei*, Krutzsch (1954) stated that *Z.h. preblei* was "averaging smaller in most cranial measurements" compared to *Z.h. campestris*. Our results refute this claim.

Subspecies/ Measurement	Number	Mean	S.D.	Min.	Max.
Z.h. campestris					
Occipitonasal length	37	23.046	0.609	21.623	24.048
Condylobasal length	39	19.944	0.571	19.083	20.92
Palatal length	39	10.105	0.305	9.313	10.635
Zygomatic length	40	9.548	0.338	8.678	10.163
Zygomatic breadth	**39**	**10.972**	**0.377**	**10.055**	**11.728**
Mastoidal breadth	**39**	**10.261**	**0.292**	**9.53**	**10.82**
Braincase breadth	40	10.321	0.263	9.765	10.7
Interorbital breadth	**38**	**4.326**	**0.176**	**3.863**	**4.833**
Upper tooth row length	40	3.689	0.14	3.365	3.945
Z.h. preblei					
Occipitonasal length	37	22.941	0.445	22.065	23.933
Condylobasal length	35	19.858	0.457	18.55	20.823
Palatal length	40	10.057	0.272	9.323	10.645
Zygomatic length	40	9.454	0.254	8.82	9.993
Zygomatic breadth	**37**	**11.193**	**0.31**	**10.52**	**12.113**
Mastoidal breadth	**38**	**10.4282**	**0.28**	**9.62**	**10.855**
Braincase breadth	38	10.345	0.211	9.81	10.838
Interorbital breadth	**40**	**4.24**	**0.145**	**3.9**	**4.495**
Upper tooth row length	39	3.725	0.112	3.418	3.97

Table 4. Qualitative morphological comparisons made by Krutzsch (1954). The left column lists the descriptions for *Z.h. preblei* and the right column list descriptions for *Z.h. campestris*. The center column (bold italics) lists the differences Krutzsch (1954) used to distinguish *Z.h. preblei* from *Z.h. campestris*.

Z.h. preblei		*Z.h. campestris*
	From topotypes of Z.h. campestris, Z.h. preblei differs as follows:	
Size medium		Size large
Color dull		(no description)
	Upper parts generally dull, averaging lighter	
Back from near Clay color to near Tawny-olive with admixture of black hair forming poorly defined dorsal band		Back from near Ochaceous-Tawny to near Ochaceous-buff with admixture of black-tipped hair forming distinct dorsal band
	less black tipped hair	
Sides lighter than back from near Clay color to near cinnamon-buff		Sides lighter than back, from near Ochaceous-buff to near yellow Ocher with black hair interspersed
	Sides duller	
Lateral line distinct and clear Ochaceous-Buff		Lateral line usually distinct, of clear Ochaceous-buff
Belly white – sometimes with faint wash of clear Ochaceous-Buff above		Belly white, usually with moderate suffusion of near Ochaceous-buff
Tail bicolored, brownish to light brownish-black above, grayish-white to yellowish-white below		Tail bicolored, brownish to brownish-black above, grayish-white to yellowish-white below
Ears dark, narrowly edged with color of sides		Ears dark, edged with Ochaceous-buff
Feet grayish-white above		Feet grayish-white above
	Averaging smaller in most cranial measurements	
Incive foramia relatively narrow and elongate		Incive foramia long and usually truncate at posterior border
	Incisive foramia narrower, not truncate posteriorly	
Auditory bullae moderately inflated		Auditory bullae well inflated
	Auditory bullae smaller, less well inflated	
Pterygoid fossae relatively broad		Pterygoid fossae broad
Postpalatal notch broadly rounded		(no description)
Interorbital region relatively narrow		(no description)
	Least interorbital constriction narrower	
Zygomatic arch not widely bowed		Zygomata relatively wide-spread and long
Frontal region well inflated		(no description)
	Frontal region usually more inflated	
Distance from incisors to postpalatal notch relatively short		(no description)

(no description)	Large medial projection on inferior ramus of zygomatic process of maxillary
(no description)	Condylbasial length and occipitonasal length relatively great
(no description)	Mastoid region and palatal region relatively broad
(no description)	Interparietal bone usually broad

Hypotheses to explain the pattern of shared mtDNAs across the range of Z.h. preblei and Z.h. campestris

1) Range of Z.h. preblei is a recent colonization from Z.h. campestris (mtDNAs represent a northward range expansion and hybridization)

2) Z.h. preblei evolved in isolation and spread north colonizing the range of Z.h. campestris (mtDNAs represent a northward range expansion and hybridization)

Reduced gene flow has led to the pattern of reduced gene flow among the range of Z.h. preblei and Z.h. campestris.

Mary M. Conner
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Logan, UT 84322 USA

April 9, 2004

Colorado Division of Wildlife
6060 Broadway
Denver, CO 80216

Dear Gary:

As I told you in my email, I am not trained in any way to evaluate specific DNA or genetic questions. My responses to your questions reflect this as do my general review comments. I first provide my review comments followed by answers to the sheet of questions you emailed.

General Comments:

Perhaps the larger issue of "What defines a species or sub-species?" is being missed in the quest for determining the legal status of the Preble's meadow jumping mouse. Ramey et al.'s report focuses on a primarily genetic, and secondarily morphometric, comparison of *Z. h. preblei* to other *Z. hudsonius* subspecies, which represents a typological view of species. Most definitions of a species include the term "does not interbreed with individuals of another species" (see Meffe and Carroll 1997 for a good discussion of the plethora of species concepts). The inability to interbreed can arise from ecological, physiological, behavioral, or physical/geographic barriers. The ability of *Z. h. preblei* to interbreed with *Z. h. campestris* needs to be addressed before the place of *Z. h. preblei* in the *Z. hudsonius* lineage can be evaluated. While Ramie et al. make a compelling argument for genetic and morphometric similarity between *Z. h. preblei* and *Z. h. campestris*, there was no evaluation of ecological, behavioral, physiological, or physical factors critical for determining taxonomic validity of *Z. h. preblei*.

The primary definition of taxonomy is "The classification of organisms in an ordered system that indicates natural relationships." Because natural relationships were not discussed, I think the title of the Ramey et al. report should be changed to "Testing Genetic and Morphometric Relationships of Preble's Meadow Jumping Mouse (*Z. h. preblei*) to Other Nearby *Z. hudsonius* Subspecies", or something similar. If the focus of the paper were clarified in the title and throughout the paper, then I would agree that Ramey et al. were justified in the conclusion that genetic and morphometric data for *Z. h. preblei* was indistinguishable from another *Z. hudsonius* subspecies, *Z. h. campestris*. However, the conclusion that *Z. h. preblei* should be lumped with *Z. h. campestris* is not warranted by Ramey's genetic and morphometric analyses. Changing the taxonomic identity of *Z. h. preblei* should not be done until determining whether ecological, behavioral, physiological, or physical barriers exist that may prevent *Z. h. preblei* from inbreeding with *Z. hudsonius*.

Finally, the "discrete" requirement that a DPS is "markedly separated from other populations of the same taxon by physical, physiological, ecological, or behavioral factors" is the key to the *Z. h. preblei* versus *Z. hudsonius* issue. If you believe that only genetic evidence should be used to define a DPS, then Ramey et al.'s assessment of *Z. h. preblei* as not worth protecting is logical. However, if you believe that more than genetics should be used to define a DPS, then Ramey et al's assessment is not logical or valid.

Specific Details:

1. I found many grammatical errors in this report; however I do not note or comment on these.
2. Throughout report – change "taxonomic differences" to "genetic and morphologic differences".
3. Page 3, paragraph 3 – The sentence "However, these authors did not gather data in such a manner as to be able to rigorously test whether *Z. h. preblei* formed a monophyletic group" needs to be changed to "However, these authors did not design their studies to answer the question of whether *Z. h. preblei* formed a monophyletic group".
4. Page 4 first paragraph (continued from previous page) and Page 10 first paragraph (continued from previous page) – Why is Crandall et al. (2000) the only criteria considered for defining a "single population"? (Note: In context, I am guessing that single population means same subspecies?) Was this agreed on before hand? Please explain the logic given that multiple definitions of species and subspecies exist (Meffe and Carroll 1997).
5. Page 5 first paragraph (continued from previous page) – The authors state that it is "critical to test whether hybridization occurs between *Z. h. preblei* and *Z. p. princeps*", but make no similar statement about the importance of evaluating whether *Z. h. preblei* can interbreed with other *Z. hudsonius* subspecies. The issue of interbreeding between *Z. h. preblei* and *Z. h. campestris* needs to be addressed.
6. Page 5 – In this methods paragraph, the authors state that they examined the literature for evidence of ecological differences, but it is not clear how or when they did this. In the last paragraph of results (page 9 first paragraph) Ramey et al. states "A review of the literature reveals that no quantitative evidence exits to reject the hypothesis of historic or recent ecological exchangeability… between *Z. h. preblei* and *Z. h. campestris*." First, what literature was reviewed? Second, and perhaps most importantly, what ecological characteristics were compared? It seems that genetic characteristics are being used as a proxy for ecological characteristics. Third, behavior, ecology, physiology, and physical/geographic factors need to be discussed as part of an taxonomic comparison. If the title and conclusions are to be left as written, then at the very least, this section needs to be vastly expanded and a table of results with literature cited produced.
7. Page 9-10 – What is a single population? Please define.
8. Page 9-10 – Basing subspecies rules on ones own work is poor scientific procedure. At the very least, provide other references for this rule or justification for this rule.
9. Page 10 – Discriminant results for a less conservative $P \geq 0.5$ rule should be included in this report for comparison to the conservative $P \geq 0.95$ rule.
10. Page 10 - A table of posterior probabilities for each specimen should be included in this report because it is a key line of evidence.
11. Page 11-12 – I agree that basing a subspecies classification on morphology of 3 specimens is not scientifically defensible. Is this really all that was done to justify *Z. h. preblei* as a subspecies?
12. Page 12 first paragraph of Conclusions section – I agree that Ramey et al. examined these 3 lines of evidence as presented. However, delete the statement in the following

paragraph (page 13) that they checked for ecological differences, which are not represented by the 3 criteria checked.

13. Page 13 second paragraph – "and the unsupported assumption that geographic isolation …" Please provide evidence that there is no geographic isolation.
14. Page 13 third paragraph – "The "discrete" requirement that a DPS is "markedly separated from other populations of the same taxon by physical, physiological, ecological, or behavioral factors" is the key to the *Z. h. preblei* versus *Z. hudsonius* issue. If you believe that only genetic evidence should be used to define a DPS then Ramey et al.'s assessment of *Z. h. preblei* as not worth protecting is logical. However, if you believe that more than genetics should be used to define a DPS, then Ramey et al's assessment is not logical or valid.
15. Figure 1 should either be in the back with other figures, or all figures should be within text.
16. Figure 2 should be reworked for a black and white printer.
17. Figure 3 is missing.
18. Table 3 – A 95% CI must be added to this table so that differences in mean measurements can be easily evaluated by the reader. Also, present SE rather than SD because what is statistically compared is the distribution of means, not the distribution of the sample.
19. Table 4 is difficult to follow visually – clean up columns and headings.
20. Page 27 – Delete hypotheses following Table 4 unless they are related to something in the text, in which case they need a to be tied via official table status.

CDOW Questions:

1. Please analyze the techniques used in the population and phylogenetic evaluation of *Zapus hudsonius Z. h. preblei* and other taxa. Were appropriate methodologies and markers used?

Morphology: The $P \geq 0.95$ rule is subjective. Ramey et al needs to present discriminant results for a $P \geq 0.5$ rule for comparison. The $P \geq 0.5$ rule is commonly used for discriminant classification (SAS 1990, Lance et al. 2000). Moreover, presenting results for both rules will provide the a full evaluation of the morphological discrimination between the 2 species. Also, it is critical that a table of posterior probabilities for each specimen be included in this report as it is a key line of evidence.

2. Are the conclusions about the taxonomic validity of *Z. h. preblei* logical and defensible as presented in the manuscript?

If you believe that genetic evidence should be used to define taxonomic validity, then Ramey et al.'s assessment of *Z. h. preblei* as not different that *Z. h. campestris* (i.e., not taxonomically valid) is logical. However, if you believe that ecological, physiological, behavioral, and geographic factors should be used to define taxonomic validity, then Ramey et al's assessment is not logical or valid.

3. Are there possible alternative interpretations of the genetics data?

Do not know.

4. Are there additional or divergent taxonomic conclusions that could be drawn from the genetics data?

Do not know.

5. Do you agree with the interpretation about possible mechanisms of reduced gene flow between *Z. h. preblei* and other subspecies of *Z. hudsonius*?

Do not know.

6. Do you agree with the concepts of Crandall et al. (2000)* for defining evolutionarily significant units?

I fully agree that evolutionarily significant units (ESU) should be defined based on both ecological data in conjunction with genetic data rather than on genetic data alone. However, the Ramey report argues for combining *Z. h. preblei* with *Z. h. campestris* based primarily on genetic data, and offers no ecological data. Citing this paper seems contradictory to the intent of the Crandall et al. (2000) paper.

7. Are there clear ecological distinctions between *Z. h. preblei* and closely related taxa that would suggest a need for specific conservation actions for this taxon?

There is no way to answer this question based on the Ramey et al. report as ecological distinctions were not discussed. To answer this, Ramey et al. should present an analysis of similarities and differences between *Z. h. preblei* and *Z. h. campestris* with respect to ecology, physiology, behavior, and geography.

Literature Cited:

Crandall, K. A., Bininda-Edmonds, O. R. P., Mace, G. M. and Wayne, R. K. 2000. Considering evolutionary processes in conservation biology: returning to the original meaning of "evolutionary significant units". Trends in Ecology and Evolution: 15(7):290-295.

Lance, R. F., M. L. Kennedy, and P. L. Leberg. 2000. Identification bias in discriminant function analyses used to evaluate putatively different taxa. Journal of Mammalogy 81:245-249.

Meffe, G. K., and C. R. Carroll. 1997. The species in conservation. Pages 57–86 *in* G. K. Meffe, and C. R. Carroll, editors. Principles of conservation biology. Sinauer Associates, Inc., Sunderland, Massachusetts, USA.

SAS Institute. 1990. SAS/STAT user's guide, Version 6. Forth edition. Volume 1. SAS Institute, Cary, North Carolina, USA.

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Specific Questions to Consider for Review of Dr. R.R. Ramey's Report on Genetic Analysis of Preble's Meadow Jumping Mouse

1. Please analyze the techniques used in the population and phylogenetic evaluation of *Zapus hudsonius preblei* and other taxa. Were appropriate methodologies and markers used?

Appropriate markers and methods were used. The control region would provide the highest possible resolution using mtDNA. As the authors state, microsatellites would provide additional insights but would not alter the general conclusion. Another mtDNA locus would also help support the conclusions (phylogenetic methods typically do better with longer sequences), but again would not change the basic conclusion. The analytical methods used are appropriate were performed quite well, in my opinion. The only additional analysis I might perform is to construct a network relationship of gene genealogies using TCS or SplitTree software. But once again, this simply allows for a different visualization of the same result. The conclusion will not change.

2. Are the conclusions about the taxonomic validity of *Z.h. preblei* logical and defensible as presented in the manuscript?

Indeed, the conclusions are right on. This work is particularly impressive by its inclusion of both genetic and morphometric data coupled with an evaluation of previous work. The author is spot on in every respect. Indeed, it looks like you will have some more work to go to figure out an appropriate taxonomy for this group. The current taxonomy clearly does not reflect the inferred evolutionary relationships. But it is clear that the *Z. H. preblei* is not a valid taxon and that the animals on the front range of CO are genetically represented in other areas.

3. Are there possible alternative interpretations of the genetics data?

I can't think of any – at least not relative to the taxonomic status of the Preble's Meadow Jumping Mouse. Some additional work could be done to develop a reasonable taxonomy and make global inferences about population structure, bottlenecks, range expansions, etc. for the species.

4. Are there additional or divergent taxonomic conclusions that could be drawn from the genetics data?

I think additional taxonomic conclusions will require additional sampling. Certainly, at the moment, I would say you have two taxa here corresponding to the two clades.

5. Do you agree with the interpretation about possible mechanisms of reduced gene flow between *Z.h. preblei* and other subspecies of *Z. hudsonius*?

Yes. The interpretations could be further substantiated by additional samples and performing a Nested Clade Analysis to partition historical demographic events from current population structure and ongoing gene flow (Templeton, 1998; Templeton, 2004).

6. Do you agree with the concepts of Crandall et al. (2000)* for defining evolutionarily significant units?

I have to say I do! I quite like that paper, as do many other folks. We have received a lot of positive feedback from it and no negative feedback that I have seen.

7. Are there clear ecological distinctions between *Z. h. preblei* and closely related taxa that would suggest a need for specific conservation actions for this taxon?

The morphological analysis suggests that there are not. If there were clear ecological differences that were persistent over evolutionary time and adaptively important, one might expect the evolution of morphological differences. In many cases, this occurs long before divergence of neutral genetic markers. For example, Polar Bears are obviously morphological distinct from Brown Bears, yet genetically they do not form distinct clades. Here we see no obvious morphological distinctiveness that relates to the designated subspecies. Indeed, the critical review of the previous work designating this subspecies identifies a number of significant problems with it. There is always a possibility that we are simply not looking at the right (critical) character. But of those examined, there does not appear to be any distinction.

In summary, I found this to be an excellent study covering all the appropriate bases. The conclusions drawn are, in my opinion, well founded and well supported by the data. The investigator has done an exceptional job in planning the study, selecting appropriate data to collect, collecting data, analyzing data, and interpreting the results. I agree with the conclusions provided by the investigator in this report and find them based on solid science.

* Crandall, K. A., Bininda-Edmonds, O. R. P., Mace, G. M. and Wayne, R. K. 2000. Considering evolutionary processes in conservation biology: returning to the original meaning of "evolutionary significant units". Trends in Ecology and Evolution: 15(7):290-295.

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7**, 381-397.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology* **13**, 789-810.

Marlis Douglas, Assistant Professor
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Colorado State University

REVIEW: Report by R.R. Ramey on *Zapus* subspecies

General problems:

- The overall tone of the manuscript lacks objectivity. Conclusions would be more convincing if data and results were presented from a less biased perspective.

- The report lacked context beyond the *Z.h.preblei* problem – the issues dealt with in this report (i.e., recent diversification of lineages) are complex and there is considerable literature available on the topic. Yet none of this was discussed.

- The molecular data are quite limited (only 355 base pairs of sequence) and these provide insufficient resolution. Thus, results are inconclusive.

- Criteria used for exclusion of particular specimens are rather unclear and seemingly subjective.

- Presentation of data is confusing and lacks sufficient and necessary detail. There are numerous typos that speak of haste in preparation.

- Manuscript is rather ambiguous with regard to various descriptions.

- Authors equate their results (a gene tree at best) with a species tree.

- Comments below often refer to (page/paragraph/line number) of the report.

1. Techniques, phylogenetic evaluation. Appropriate methods and markers?

Yes and no. The overall approach seems appropriate. However, there are a number of issues that are not addressed in the manuscript, some of which may substantially impact results. Details that support results and conclusions are lacking. Additional and appropriate analyses could have been performed for both molecules and morphology.

Molecular work:

DNA extraction was appropriate. DNAeasy Kit from Qiagen is known to produce clean DNA from difficult samples. PCR amplifications are appropriate, although 200-300 ng of DNA in a 25 ul reaction volume seems large, but might be necessitated by low quality (i.e., low molecular weight) of extracted DNA. It is also appropriate to sequence the target region in both directions, obtaining sequences for both strands.

Museum specimens are indeed a valuable resource in that specimens from a large geographic area can be made available, and a study can thus be executed in relatively short time. However, the quality of DNA extracted from museum specimens is often inferior. It is most often fragmented and consists of small pieces in low quantity that

makes it difficult to reliably amplify target sequences (Pääbo 1989). Thus, primers should be designed from sequences obtained from fresh tissues, and must be selected to produce short products approximately 100—200 base pairs in length (as per Drew et al. 2003). The problem is that this procedure requires up to 5x more PCR amplifications than normal, which in turn increases cost and reduces sample sizes. However, it does sample a large number of overall base pairs, which is important. This apparently was not done in the present study.

Authors allude to some of these problems (page 6) and in fact developed internal primers to amplify difficult samples using nested PCR. However, cross-contamination is an issue with such "ancient DNA" samples. While DNA can be amplified from minute amounts of tissue using forensic techniques, contamination of such templates with high quality DNA from other samples is a major concern. Another aspect is that PCR can incorporate the wrong base during replication; if such a mistake is incorporated early in PCR cycles, it will be reproduced in all subsequent cycles. Again, this is a major issue for ancient DNA samples where little template DNA is available to start the reaction. One way to address this issue would be to generate independent replicate amplifications/sequences of samples so as to calculate genotyping error. This is particularly important if haplotypes differ by only a single base, as is seemingly the case with the present data set.

One must assume that authors took all necessary precautions to avoid contamination of their samples, but it would certainly be more convincing if indeed they explicitly stated in their lab procedures the manner in which they dealt with these issues.

Descriptions are often vague. For example, authors state (6/2/1-2) that some DNA extracts did not amplify well, but there is no information on how many? It is also not clear how they could get amplified DNA when the initial PCR did not amplify at all (6/2/2)?

Another more substantial problem encountered when working with ancient DNA is the size of fragments (i.e., numbers of base pairs) that can be reliably amplified and sequenced (as noted above). The current analysis is based on 355 base pairs (bp) of sequence data - this is a marginal data set for population-level analyses judging from today's standards. As a general rule, at least 1,000 bp should be evaluated to substantiate findings and make results conclusive. An analysis of several independent molecular markers that corroborate findings would also make the study more convincing.

The control-region (or D-loop) is generally a good marker to examine recently diverged taxa because it has a high rate of evolution. Presumably *Z.h.preblei* became isolated post-pleistocene (6/2/9), yet a time span of 10,000 years is about the limit for mtDNA resolution. Taxa that are more recent diverged would be difficult to detect via mtDNA analysis. The control-region does not code for a protein which explains its fast rate of evolution, but this is also a drawback in that it limits the types of analyses that can be done. For example, those that rely on codon position cannot be utilized.

In addition, it is not at all clear which section of the control region was sequenced. Here, site positions should be provided relative to a standard sequence available from GenBank. The control-region consists of rather variable segments at the 5' and 3' end, and a relatively conserved middle region. Since nested primers are designed internally from the flanking regions, it is likely that the region sequenced in this study straddles the conserved middle segment, and thus encapsulates only a moderate amount of genetic diversity. This is an issue in that the analyzed fragment does not provide sufficient resolution to determine interrelationships of the taxa under study (see below). The question then becomes, is the lack of variation due to similarities among OTUs, or is it instead a function of the conservative (and limited) nature of the molecular marker?

Molecular data analyses

Sample sizes are appropriate (Crandall et al. 2000). Phylogenetic analyses seem appropriate. Standard procedures were used to generate phylogenies, and data were first examined via ModelTest so as to determine the model of sequence evolution. However, details are lacking for the AMOVA. Were alternative genetic structures tested for significance?

In addition, the text is confusing and it is not at all clear how many samples were indeed used for analyses. On page 6 (6/1/1/), authors state that 151 sequences were aligned, whereas the heading for Fig. 2 indicated that analyses are based on 176 samples. The sum of all specimens listed per haplotype for the ingroup (i.e., *Z.hudsonius*) is 151.

Also, the basis for exclusion of specimens that showed haplotype characteristic of other subspecies is rather vague (page 8). Why is it reasonable to assume that those *Z.h.campestris* with haplotype L/Pal/C-2 were misidentified and can thus be excluded from analyses, whereas those *Z.h.campestris* showing haplotype C/P-1 through 4 are not? Details on collection data are also lacking. How reliable are the locality definitions? Further, why wasn't the identification of these specimens confirmed by re-examination? Authors state that this is a strong suit of voucher specimens.

Why is a Neighbor joining phylogram presented, instead of an MP, ML or BA tree? Authors state that other analysis produced "similar" trees, but phylograms of these should be provided so that tree topology and nodal support can be examined.

A haplotype network or minimum spanning tree of haplotypes would also be informative. The shallow terminal branches of the phylogram suggest that haplotypes differ by single base pairs. Further, haplotype diversity statistics and an appendix showing haplotypes/variable sites should be provided (see also comment under Point 3).

It is not clear which samples were used to calculate nucleotide diversity. High nucleotide diversity in *Z.h.campestris* and *Z.h.preblei* could be due to divergent and "mis-identified"

individuals, as suggested by the high standard deviation (8/5). Again, it's not clear whether these samples were included in the calculation of nucleotide diversity.

Morphometric analyses

There are several perceived difficulties with the morphometric analyses.
(10/2/3) – "In several cases, fewer measurements were taken because of breakage or not taken because of previous breakage." These should be enumerated in the report, along with the museum numbers of the specimens excluded. If this is not done, then how will another scientist be able to replicate the study?

Discriminant analysis is an inappropriate multivariate procedure for this study in that it requires that specimens be *a priori* allocated to group. If indeed one is testing for group membership, then pre-allocation to group biases the study. Given the ambiguity of specimen assignment in the molecular analyses, a more effective means of evaluation would have been a principal components analysis of morphometric data based on the variance-covariance matrix. Data should also be first tested for normality.

(10/3/10—11) – "Males and females were pooled in the analyses because of a lack of cranial sexual dimorphism in *Z.princeps* and *Z.hudsonius* (Connor and Shenk 2003)." However, Connor and Shenk evaluated *Z.h.preblei* and *Z.p.princeps* whereas the current report evaluated *Z.h.preblei* and *Z.h.campestris.* If indeed the object is to test the sexes of two subspecies for potential differences in morphology, then one should not apply as the test those results previously generated for different subspecies. Additionally, if the object is to evaluate group membership (in this case, sex) using morphological criteria, then pre-allocation to group would again bias the analysis (as per caveats regarding discriminant analysis above).

It is also somewhat confusing that other subspecies of *Z.hudsonius* were not examined morphologically as well. And since the researchers went to all the trouble to measure their specimens, why did they not take other (additional) standard morphometric measures?

(11/1/8) – "…only larger for one measurement….and it was only marginally significant (P=0.037)." Again, a value judgement that undermines the objectivity of the study.

2. Are conclusions about taxonomic validity of *Z.h.preblei* logical and defensible?

I personally cannot follow the logic. If *Z.h.preblei* and *Z.h.campestris* should be synonymized based on shared haplotypes, then other *Z.hudsonius* subspecies must be synonymized as well. This logic could even be extended to *Z.hudsonius* and *Z.p.princeps*, since haplotypes of the latter were found within *Z.hudsonius*. This suggests either a very complex taxonomic problem confounded by quite recent (i.e.,

post-Pleistocene) diversification, or a problem with the resolution of the molecular marker (as above).

The limitations of the data affect resolution of analyses and thus render results inconclusive. Relationships among haplotypes are not (or only poorly) resolved in the neighbor joining tree (Fig. 2). Additional sequence data from fast evolving, independent markers are needed (as recommended by Haig 1998). Data based on a different marker might still remain incongruent, but that in itself reveals important aspects of the phylogenetic history of a species (Hey et al. 2003).

Not clear why *Z.p.princeps* was selected as outgroup. The work by J. Cook is unpublished and thus unavailable for evaluation. Monophyly does not render one taxa as outgroup for another.

It is not clear why hybridization between *Z.h.preblei* and *Z.p.princeps* should invalidate the taxonomic status of *Z.h.preblei* (3/4/5-7)? The biological species concept (BCS) uses reproductive isolation as a criterion of demarcation, but it is generally recognized that the ability to hybridize is a pleisomorphic (i.e., ancestral) trait that offers little with regard to recent diversification of species.

Museum collection data (e.g., date of collection, precise collection locality etc.) should be provided in an Appendix. This would clarify the validity of original identifications and also provide further information about DNA quality in that reviewers could judge the ages of various samples.

Conclusions based on AMOVA are not justified. High percentage of within vs among subspecies diversity is influenced by resolution of the marker and demographics of the population (e.g., bottlenecks, population fluctuations, effective population size, etc.).

The criterion of “greater genetic diversity among putative taxa than within” (8/5/1—4) is a flawed concept. Genetic diversity is dependent on population size and population history. Paetkau (1999) emphasized that population demographics do influence retention of genetic diversity, including ancestral haplotypes and time to complete lineage sorting (or reciprocal monophyly).

3. Alternative interpretations for genetic data?

Identical haplotypes in *Z.h.preblei* and *Z.h.campestris* could be explained by:

- Retention of ancestral polymorphism and incomplete lineage sorting

- Homoplasy (similar character state but independent evolutionary origin)
- Genotyping error

Pattern could also be explained by retention of ancestral polymorphism in *Z.h.preblei* and *Z.h.campestris*. In other words, the detected variation stems from mutations that occurred prior to their divergence, and which is still retained in both subspecies. The marker does not provide enough resolution to differentiate the two lineages and the data suggest incomplete lineage sorting at this level of resolution. A more extensive data set based on markers with appropriate evolutionary rate might reveal additional mutations that are not shared between the two subspecies. It is important to note that there is a clear frequency difference regarding the identities of individuals contained in the four *Z.h.preblei* haplotypes (C/P1—4). Only one or two specimens of *Z.h.campestris* are found within these haplotypes, with the majority of individuals (90%, 89%, 86% and 82%) being *Z.h.preblei*. If indeed *Z.h.preblei* simply represents a recent range extension of *Z.h.campestris*, then one would expect to find *Z.h.preblei* haplotypes scattered throughout the *Z.h.campestris* clade. The fact that *Z.h.preblei* haplotypes cluster together suggests they diverged from one another and thus underscores the argument that *Z.h.preblei* might be on its own evolutionary trajectory.

Paetkau (1999) raised concerns about applying purely genetic identification criterion for an ESU and pointed out that population demographics do indeed influence time to complete lineage sorting. Even low frequency haplotypes are retained in large populations over sustained time, whereas small populations lose genetic diversity randomly through drift.

An alternative although less likely hypothesis is that the shared haplotypes arose independently in both subspecies thus representing homoplasy rather than homology.

As alluded to above, genotyping error is a concern with museum-based molecular studies. Independent replicates (including DNA extraction, amplification and sequencing) would corroborate findings or reveal genotyping errors. Again, more information on haplotypes would help to minimize this problem.

There are various analyses that could be performed differently or in addition to the ones presented. For example, AMOVA could be used to test hypotheses of alternative genetic structure, and data could be examined for an isolation-by-distance effect. Most importantly, a Nested Clade Analysis would enable separation of historic and demographic events (as suggested by Crandall et al. 2000). This is particularly important to verify the hypothesis of “founder effects and range expansion” (9/2/8-9). Such analyses helped to clarify genetic structure in fragmented populations of another small mammal with a controversial conservation history (Swei et al. 2003).

The assumption that microsatellite DNA loci will provide less resolution than mitochondrial DNA sequences (5/1/1-4) is completely erroneous. It is also unclear why *Z.h.preblei*, if shown to be distinct based on mtDNA sequence data, must then be tested for hybridization, and furthermore, why microsatellite DNA loci should be used for this task (nuclear markers yes, but preferably not microsatellite DNAs).

4. Additional or divergent taxonomic conclusions based on these data?

Alternative conclusions are most certainly possible. However, limitations of the genetic data hamper any conclusions, and render as speculative any taxonomic interpretations.

Shared haplotypes between *Z.h.preblei* and *Z.h.campestris* could simply be due to a lack of resolution for the limited data set. Further, incomplete lineage sorting is to be expected given the presumed time frame of divergence. This is particularly so if a population is large or expanding (*Z.h.campestris*) and if ancestral haplotypes are retained (Paetkau 1999). However, the presence of different haplotype frequencies suggests that *Z.h.preblei* is on its own trajectory and could warrant DPS ("distinct population status") if indeed corroborated with a more comprehensive genetic evaluation.

It also seems as if the report confuses a "gene tree" with a "species tree." As a means of explanation, a phylogeny of a species represents a multitude of nested component trees, each reflecting the history of populations as determined by single characters. When a component tree is derived from DNA information (e.g., haplotype sequences) it is referred to as a "gene tree" (Avise 2000). On the other hand, a "species tree" can be viewed (Avise 1994:126) as "…a single pedigree that extends [historically] as an unbroken chain of parent-offspring genetic transmission…." Hence, species trees are histories of organisms (i.e., pedigrees) whereas gene trees are histories of single traits (Avise 2000). The distinction is important.

In phylogenetic reconstruction, one must understand that a gene tree does not necessarily reflect a species tree. Not only can gene trees differ in their topology one from another, but also from species trees. Differences are due to a variety of biological factors (e.g., stochastic lineage sorting, introgressive hybridization, horizontal transfer, etc.; Avise 1994, 2000). Yet a common practice is to use gene trees as an estimate of the species tree (as herein). This is a tenuous association at best, because there are ample reasons for gene trees and species trees to be discordant (as above). This is particularly true in the present report because the gene tree is based on very few base pairs of data. Thus, the original hypothesis carried the caveat "distinguishable….using mitochondrial DNA sequence data" ((4/2/6), yet this caveat was somehow dropped in the conclusions and the gene tree then became instead the species tree for the taxon.

5. Interpretations about possible mechanisms of reduced gene flow between *Z.h.preblei* and other subspecies of *Z.hudsonius*?

I also cannot follow the logic of the argument that "founder effect and range expansion" contradict evidence of restricted genetic exchange. I would agree that low haplotype diversity in *Z.h.preblei* suggests a population bottleneck. But if it was due to a recent founder event, one would expect the haplotypes in *Z.h.preblei* to represent various *Z.h.campestris* haplotypes and not just those that form a distinct cluster.

The existence of four very similar haplotypes suggests a bottleneck within *Z.h.preblei* populations followed by subsequent expansion with low effective population size. It also indicates a lack of (or at least reduced levels of) gene flow between *Z.h.preblei* and other *Z.hudsonius* subspecies. Hey *et al*. (2003) argued that bottlenecks will obscure genetic divergence among populations, even within such ancient lineages as Tuataras. Based on the data in this study, it appears that the Front Range population is of "recent" (i.e., post-Pleistocene) origin. Again, the molecular data are limited in their number and capabilities, and thus do not provide sufficient resolution from which to draw conclusions. Additional genetic data are needed.

Thus, genetic divergence originating through range expansion and subsequent reduced gene flow is indeed a potential mechanism for speciation. For example, Abbott and Double (2003a,b) used mitochondrial and microsatellite DNA to discover that shy albatross arose from white albatross through range expansion.

6. Crandall et al. (2000) definition of ESU?

The definition for ESUs as provided in the report is rather vague. For example, Mortiz (1994) included significant differences in nuclear alleles as an additional criterion for designation of an ESU.

Moritz (1994) also emphasized the distinction between the biological definition of ESU (and MU) and the genetic criteria. He argues that "the term 'significant' in ESU should be seen as recognition that the set of populations has been historically isolated and accordingly, is likely to have a distinct potential" (Mortiz 1994:373). Further, his genetic criterion for recognizing and ESU includes mitochondrial and nuclear loci: "ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci." (Mortiz 1994:373).

Ecological exchangeability is one criterion proposed by Crandall et al. (2000) and this suggestion has merit. However, its application is hampered by lack of ecological data for most rare species. Pacific salmon are probably a notable exception. However this report does not provide data (or even references) concerning the ecological characteristics of the subspecies under study. Did I miss something here?

Further, Crandall et al. stressed that "failure to reject the null hypothesis does not imply that the null hypothesis is true, but could simply be a result of the lack of relevant data" (Crandall et al. 2000:293). I would argue this is indeed the case here.

7. Clear ecological distinctions between *Z.h.preblei* and closely related taxa?

Authors state that they "examined literature for evidence of ecological differences between subspecies" (5/2/6-7), yet they do not provide a single reference. Also, additional morphological data might provide some insight?

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Review of Ramey et al., Testing the taxonomic validity of Preble's Meadow Jumping Mouse (*Zapus hudsonius preblei*)

Ramey et al. employ the appropriate methods, markers, evidence, and interpretation to convincingly argue that *Z. h. preblei* is not a valid subspecies, and should be synonymized under *Z. h. campestris*. I think that the ESU is an appropriate and useful genetic unit that should be employed by conservation agencies as well as phylogeographers. The study by Ramey et al. has several small editorial errors and reference omissions (e.g., Hafner et al. 1981 and Hafner 1997 are cited as Hafner 1981 and Hafner 1987 in the second paragraph), and the tone is unnecessarily ponderous, condescending, and preachy. However, I agree with all of the systematic and taxonomic conclusions, and would also encourage regulatory agencies to employ systematists to provide such systematic reviews wherever it is practicable. I think it's rather absurd to consider regulatory agencies to be responsible for supporting in-depth systematic studies of this sort for every taxon under consideration, but an accurate taxonomy a laudable goal.

There remain several confusing aspects to the mtDNA data, but none that would alter the overall systematic and taxonomic conclusions. Specifically, Ramey et al. list a locality in Kansas as "Macon Co."; there is no Macon Co. in Kansas (could it be Marion Co.?). On page 8 they state that two sequences from Clay Co. were more similar to *campestris* than to *pallidus*, and then say that "they" (these two plus one presumed *hudsonius* that turned out to be a *princeps*) were "presumed misidentified and thus not included." I understand why *princeps* that were clearly misidentified as *hudsonius* were not included, but isn't it more likely that either a *campestris* mtDNA clone somehow remained or has found its way into *pallidus*, or (even more likely) there was some cataloging (museum) or experimental (laboratory) error, and the Clay Co. specimens are actually from *western* South Dakota. Given the "Macon Co." error, that seems to me to be the best bet. By the way, the Clay Co. sequences *were* included in the Neighbor-joining tree, so from what were they excluded, the Table?

While I support the taxonomic interpretations of Ramey et al., I disagree strongly with their implied conclusion that synonymy with *campestris* automatically translates into conservation security for the geographically expanded taxon. Yes, the expanded subspecies is "more common and widespread than previously thought," but that does not necessarily mean that the new taxon is secure, or that this represents a "misallocation of scarce conservation resources to populations that are not genetically or ecologically unique." Here Ramey et al. went well beyond their data, and failed to consider the conservation status of *campestris*. It would have been quite simple for Ramey et al. to

consult the IUCN Status Survey and Conservation Action Plan for North American Rodents (Hafner et al. 1998; http://www.iucn.org/themes/ssc/actionplans/northamericanrodents/5geo.pdf). In the section on *Zapus hudsonius*, Hafner and Yensen (1998) consider *preblei* to be Endangered (EN): B1; B2c (IUCN Red List Category; IUCN 1994), but also consider *campestris* to be of concern: Vulnerable (VU): B1; B2c.

> EN: B1; B2c = Endangered, facing a very high risk of extinction in the wild in the near future; extent of occurrence estimated to be less than 100 km^2, and estimates indicating: B1) severely fragmented; and B2c) Continuing decline, observed, inferred, or projected, in area, extent, and/or quality of habitat.
>
> VU: B1; B2c = Vulnerable, facing a high risk of extinction in the wild in the medium-term future; extent of occurrence estimated to be less than 100 km^2, and estimates indicating: B1) severely fragmented; and B2c) Continuing decline, observed, inferred, or projected, in area, extent, and/or quality of habitat.

Overgrazing and loss of riparian habitat has been implicated as the major deleterious impact on populations of *campestris* in Wyoming, South Dakota, and Montana (Hafner and Yensen 1994). Thus, although the expanded *campestris* enjoys a larger geographic range, it (including populations previously assigned to *preblei*) is of conservation concern throughout its range.

Moreover, mapping of the populations studied by Ramey et al. (see my Fig. 1) puts their Neighbor-joining tree in a geographic context, and allows phylogeographic interpretation. Due to the closer similarity of mtDNA clones of *luteus* and *pallidus*, it is apparent that the expanded *campestris* diverged prior to the geographic isolation of *luteus* from *pallidus*, which itself probably was associated with drying of grasslands habitat during the Hysithermal (6000 yBP) following the Wisconsinan glaciation. Thus, *campestris* may have been isolated from the main distribution of *hudsonius* during the Wisconsinan full-glacial. In any event, Ramey et al. clearly indicate that *campestris* is genetically more distinct from *pallidus* than is *luteus*, and so deserves more consideration as a unique gene pool.

Twenty years ago, *Z. h. luteus* was not only believed to be *Z. princeps luteus,* but also was considered to be extinct from the Rio Grande Valley of New Mexico. More recently, *Z. h. preblei* was considered to be on the verge of extinction. Conservation concern led to targeted fieldwork, which in turn led to the discovery of additional populations of *luteus* in the Rio Grande Valley and in southeastern Colorado, and of *preblei* along the eastern edge of the Rocky Mountains. In my opinion, *Z. h. luteus* is relatively secure and not currently threatened, but I believe that *Z. h. campestris* (including *preblei*) remains imperiled.

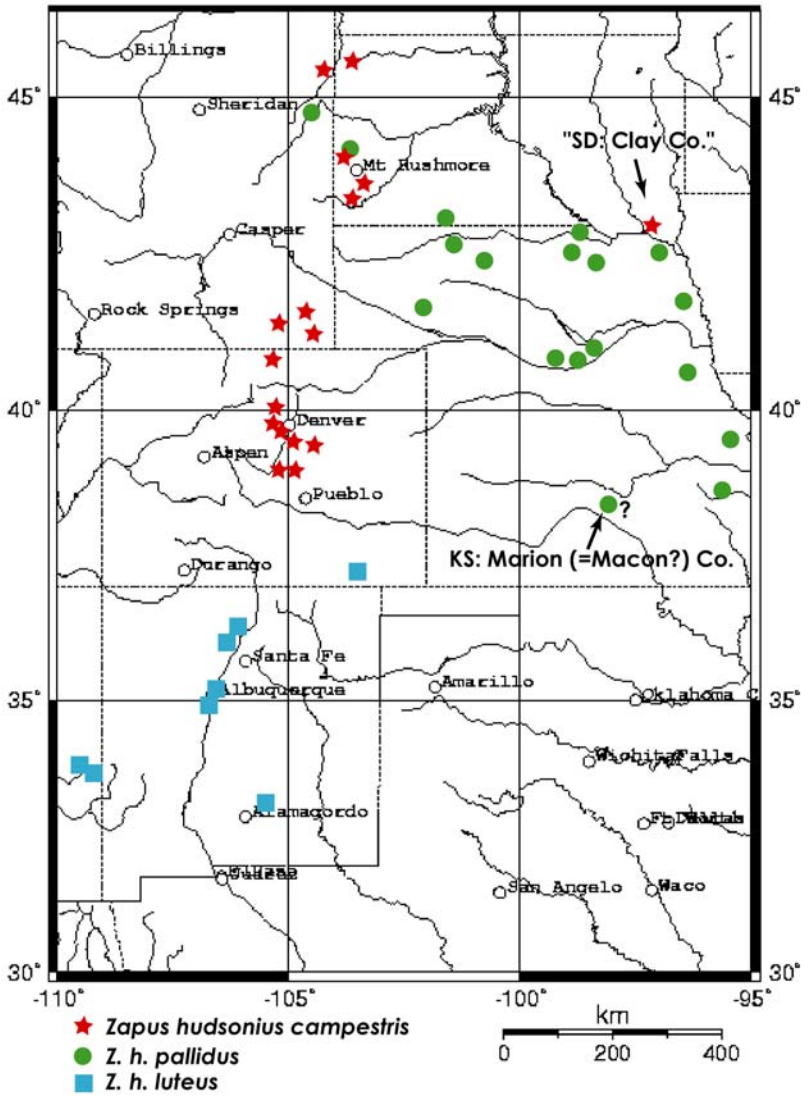

Fig. 1—Distribution of mtDNA samples included in Ramey et al., indicating their assignment into *Z. h. pallidus, Z. h. luteus,* and *Z. h. campestris* (including populations formerly assigned to *Z. h. preblei*).

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30 March 2004

Gary Skiba
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Dear Gary:

In this letter I present my review of the manuscript titled **Testing the Taxonomic Validity of Preble's Meadow Jumping Mouse (*Zapus hudsonius preblei*)** by R. R. Ramey II, H.-P. Liu, and L. Carpenter, revised 12 March 2004.

I find this paper very clear in its presentation, use of hypothesis-testing, and overall good science. The evidence suggests that *Zapus hudsonius preblei* is not genetically or morphologically distinct from *Z. h. campestris*. I am not a geneticist, and will limit my subsequent comments to the other parts of the paper.

The morphometric data and analysis appear solid. Krutzch (1954) acknowledged that *Z. h. preblei* most closely resembled *Z. h. campestris* and the techniques he used were the best available science for his time. Also, it was recently brought to my attention that a more recent study of the systematics and biology of the genus *Zapus* found insufficient morphological evidence to support subspecific status for *Z. h. preblei* (Jones 1981, as cited in Beauvais 1998).

While I agree that the qualitative descriptions can be vague and impossible to reproduce exactly, there is one character used by Krutzsch (1954) that may be straightforward to see and evaluate: The "incisive foramina long and usually truncated at posterior border" on *Z. h. campestris,* compared to "incisive foramina narrower, not truncate posteriorly" on *Z. h. preblei.* However, I consider this a moot point because the overall analysis clearly indicates a lack of morphological distinction between the two taxa.

The interpretation of reduced gene flow attributed to a southern colonization event seems quite plausible. And that hypothesis is preferred to the isolation of *Z. h. preblei* with a northward colonization and subsequent hybridization with *Z. h. campestris* because it is a more parsimonious explanation.

A southern colonization from the Black Hills to southeastern Wyoming could be very difficult through Thunder Basin due to dry conditions and the fact that the drainages run east-west, thus requiring the crossing of drainages and ridges and rendering movement difficult for a mouse. Perhaps a better avenue occurs along the north-south axis created by the Powder River on the east flank of the Bighorns, and the Belle Fourche, the drainage on which *Z. h. campestris* specimens were collected at Bear Lodge. Cooler, wetter conditions during two events in the Neoglaciation period in the Rocky Mountains may have provided an opportunity for such movement along these drainages in the past 900 years or so. Most probably there are no trapping or collection data from these areas, a considerable gap in our knowledge. Interestingly, the indication that the four Albany County *Z. h. preblei* specimens were genetically *Z. princeps* removes some of the northernmost *Z. h. preblei* specimens from the picture.

I find the paper by Crandall et al. (2000) to be a clear treatment of evolutionary significant units (ESUs). I strongly agree for the need to incorporate ecological data, as well as morphometric data, along with genetic data. I find the broader categorization of population distinctiveness to be more realistic and appropriate than a dichotomous view because it is better able to address the complexity nature presents. In the paradigm presented by Crandall et al. (2000), *Z. h. preblei* (or *Z. h. preblei* and *Z. h. campestris* combined) may fit case # 6.

Although there appears to be a lack of readily-available published information, I find the important question of potential ecological differences between the two taxa, or between the combined *Z. h. preblei* and *Z. h. campestris* and the remaining subspecies, nonetheless unanswered. The Front Range likely has less moisture than the Black Hills area, and the combined Front Range/Black Hills area has less moisture than the more eastern range of the species. The potential ecological (and associated behavioral) uniqueness of *Z. h. preblei* in being restricted to riparian habitats is worthy of further investigation, and a better understanding of how *Z. h. campestris* fits into this ecological paradigm is of considerable interest. In the past 10 years or so, the recognition of the importance of animal behavior to conservation biology has grown (e.g., Caro 1998, Festa-Bianchet and Apollonio 2003).

I recognize that, following the guidelines of Crandall et al. (2000), ecological exchangeability should be demonstrably heritable. This may be difficult to show for the potential habitat differences described above. Furthermore, Crandall et al. (2000) indicate that in their review of 98 studies, ecological data were frequently lacking.

I think the status of *Z. h. campestris* now becomes an important biological question, as very little is known about this subspecies. This taxon is categorized as vulnerable by the International Union on the Conservation of Nature. Thus although *Z. h. preblei* may not be distinct, there is the possibility that the two subspecies together may be imperiled.

Conversations with Gary Beauvais of the Wyoming Natural Diversity Database were of benefit to me in developing a better understanding of certain elements of the Wyoming

landscape and for the Jones (1981) reference.

Please let me know if you have any questions.

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Review

Testing the Taxonomic Validity of Preble's Meadow Jumping Mouse (*Zapus hudsonius preblei*)
Ramey, R. R. II, H.-P. Liu, L. Carpenter

Submitted by Jeff Mitton to Gary Skiba

This study examines mtDNA sequence data and skull measurements of Preble's meadow jumping mouse, *Zapus hudsonius preblei*. The mtDNA sequences are compared to sequences from *Z. h. luteus*, *Z. h. pallidus*, *Z. h. campestris*, and the outgroups *Zapus princeps idahoensis*, *Z. p. princeps*, and *Z. p. utahensis*. Skull measurements are compared between *Z. h. preblei* and *Z. h. campestris*. Two important results emerge from these studies:

1) the haplotypes detected in *Z. h. preblei* are a subset of the haplotypes in *Z. h. campestris*--that is, the samples of *Z. h. preblei* did not reveal any unique haplotypes;

2) a discriminant function of skull measurements could only correctly classify 48% of the individuals to their correct subspecies—about the percentage (50%) that could be correctly assigned by random guessing.

Specific comments

Abstract line 3: change to analysis of skull measurements from 80…

Abstract line 6: change *campestris*, all to *campestri*s; all

Page 3 change then previously to than previously

Page 3, 4th line up from the bottom. Hybridization with *Z. princeps* seems to come out of left field, and then is not mentioned again. Is this hybridization documented or speculated about in the literature? Why is it mentioned here? Will it be assessed in this report?

Page 5, first line—it will be unlikely that it will be differentiated for nuclear microsatellite DNA. This reviewer disagrees. The evolutionary rate of mtDNA is about 5 to 10 times as fast as nuclear genes in general, but nuclear microsatellites are a special case, for their mutation rates are far higher (in humans, frequently in the range .05 to .001) than rates in mtDNA. MtDNA should differentiate faster than most nuclear genes,

but microsatellites should differentiate faster than any other genetic marker.

Page 5, first paragraph: Once again, hybridization with *Z. princeps* is mentioned—this is confusing to the reader, who has not yet been given any explanation for considering hybridization.

Page 5, second paragraph—Is it sufficient to mention the crosshair classification described in Crandall et al (2000), or does this report need a brief description of the classification?

Page 5, last paragraph—The sequence of the primers should be included here. Alternatively, a reference to these primers should be given (preferably, both)

Page 6, second paragraph—same as the preceding comment

Page 7 The first two full sentences on this page are redundant

Page 8 Four *Z. h. preblei* were removed from this study; their mtDNA haplotypes were more similar to those of *Z. princeps princeps*, and the authors assumed that they were misidentified. (Or perhaps this is why hybridization with *Z. princeps* has been mentioned). This assessment should be given fuller treatment, for the deletion of *Z. h. preblei* haplotypes might cause some suspicion. One way around this is to include them in a tree, to show the critical reader that they are far away from *Z. h. preblei*, and in a clade with *Z. princeps*, supported by bootstrap values. Then the authors can assert that the samples were misidentified, or, alternatively, the *Z. princeps* haplotypes record hybridization with *Z. h. preblei*. The other deletions of data are less critical, for those subspecies are not being evaluated here.

Page 9, last full paragraph. This reviewer agrees. The most parsimonious assumption is that *Z. h. preblei* is simply an arm of the distribution of *Z. h. campestris*, and therefore contains a subset of the variation in *Z. h. campestris*.

Page 13, first line. “Jthe lack of genetic, morphological or published ecological evidence for genetic distinctiveness (including adaptive divergence)…” It sounds like there have not been any comparative studies of the life history variation or habitats of *Z. h. campestris* and *Z. h. preblei*. Is that the case? Either way, the conclusion is not changed, but it provides more information to the critical reader to report that “no studies have been performed” versus “comparative studies have not revealed differences.”

Page 13 “significant gap in the range of a taxon…”. This could not be evaluated by this reviewer, for the copy of the report contained only figure 2, no figure 1, no figure 3.

Specific Questions

#1. Yes, appropriate markers and methods were used.

#2 I have suggested some revisions, including a more explicit treatment of the 4 *Z. h. preblei* removed from this study. If it is generally agreed that those 4 samples contained mtDNA from *Z. princeps*, then yes, I believe that the conclusions in this report are logical and defensible.

#3 If the removal of the 4 samples mentioned in #3 is prudent and appropriate, then I do not think the data have another parsimonious explanantion.

#4 Additional observations: either misidentifcation of species and subspecies is not uncommon, or hybridization among taxa is not uncommon. These hypotheses could be tested with nuclear markers.

#5 I did not note any interpretation of the mechanism of reduced gene flow. In fact, Ramey et al reported that all of the haplotypes in *Z. h. preblei* were found in *Z. h. campestris*. Note that the figure of geographic distributions was not included in the review copy, so this reviewer could not assess or appreciate the proximity of the geographic distributions

#6—Do you agree with the concepts of Crandall et al (2000) for defining evolutionary significant units?
In general, I do agree. The reliance on both genetic and ecological exchangeability has a lot of biological intuition behind it. Unfortunately, the number of studies considering substantial genetic and ecological data are really quite small. For example, in the present study of *Zapus*, that all populations are adjacent to streams, and that "A review of the literature reveals that no quantitative evidence exists to reject the hypotheses of historic or recent ecological exchangeability…". That is, no one has reported adaptive or ecological differences, but it is not clear that anyone has looked. Thus, we can fill in minus signs on the upper and lower right of the crosshair classification (Table 1 and Figure 1 in Crandall) but I think these are the necessary presumptions that you make in the absence of rigorously collected, comparative data.

#7 No.

submitted by Sara Oyler-McCance
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Review of "Testing the Taxonomic Validity of Preble's Meadow Jumping Mouse (*Zapus hudsonius preblei*)" Overall, I agree with the authors' approach to investigating the taxonomic validity of Preble's Meadow Jumping Mouse. Specifically, I believe it is a good idea to use multiple lines of evidence (not just genetic data) to clarify taxonomic borders. Typically these lines of evidence are genetics, morphology, and behavior (and sometimes geography). The authors do present genetic and morphological evidence and speak of ecological or perhaps behavioral evidence although this is not well defined in this report. My comments will focus more on the genetic aspects of this study than on the morphological aspects as is consistent with my experience and expertise.

From a genetic standpoint, this study uses an appropriate genetic marker (mtDNA sequence data) and does an excellent job analyzing the data from a phylogenetic standpoint. I see no problems with the sampling scheme, the technical aspects of the lab work, the appropriateness of the marker used, or the phylogenetic analysis. This study provides a great data set from which to **begin** to answer the question at hand. I do not feel, however, that this study by any means resolves the taxonomic question. Further, I feel that some of the conclusions made by the authors are debatable.

In the literature there exists a huge controversy about how to define a species that has resulted in a myriad of different species concepts (Biological Species Concept, Phylogenetic Species Concept, Evolutionary Species Concept, etc.). Trying to define a subspecies is even more nebulous but has resulted in a similar discussion of how to define a "unit" for conservation below the species level. Several authors (mainly Moritz) have described an Evolutionary Significant Unit (ESU) based purely on genetic data and indicate that a valid ESU must be reciprocally monophyletic. Others suggest different concepts including the one (Crandall et al. 2000) used in this report. My point here is that there is not one "accepted" definition in the literature of how to define a subspecies or even a species. How you delineate the boundaries of a subspecies depends upon which definition or groups of definitions you use. Different species or ESU concepts can be applied to the same data with widely different results. For example, in 2000, the AOU recognized the Gunnison Sage-Grouse as a new species based on the Biological Species Concept. Gunnison Sage-Grouse exhibit differences in behavior and morphology and are reproductively isolated from Greater Sage-Grouse. Genetic data from that study show a lack of gene flow between the two species and mtDNA haplotypes and nuclear microsatellite alleles that are unique to the Gunnison Sage-Grouse. However, if you apply Moritz's criterion of reciprocal monophyly, the Gunnison Sage-Grouse do not even qualify as an ESU.

The authors of this study use three criteria to determine whether or not the Preble's subspecies is a valid one from the genetic standpoint: reciprocal monophlyly, Ramey's AMOVA test, and the criterion of Crandall et al. 2000. The author's state in this report that they feel the reciprocal monophyly definition is too strict. I agree with this idea particularly in light of my experience with the genetics of Sage-Grouse. The second test is Ramey's assertion that the subspecies boundary exists when there is more variation among groups than within groups using AMOVA analysis. This measure, while discussed in 3 papers published by Ramey is not well tested in the literature or accepted as a standard measure. The authors make the statement that this measure is less restrictive than the reciprocal monophyly definition and I am not sure that in all cases it really is. I would like to see a critical review of this measure before I would use it as a standard. Finally, the authors use the criterion put forth by Crandall et al 2000. I agree that this criterion conceptually is a good one but may be more difficult to apply with empirical data.

In this study the authors find that *Z. h. campestris* is most closely related to *Z. h. preblei* and that *Z. h. luteus* is most closely related to *Z. h. pallidus*. Further, they show that all four haplotypes found in *Z. h. preblei* are shared with *Z. h. campestris.* This does seem to suggest that somehow *Z. h. preblei* are a subset of *Z. h. campestris.* At this point, I wished the authors had presented a map showing the range of each subspecies and where the corresponding haplotypes were found. I tried to do this using their data from Table 1. From what I could understand from this table, it appears as though all the *Z. h. campestris* samples from Custer, SD shared haplotypes with *Z. h. preblei*. Thus, there are no haplotypes in Custer, SD found so far that belong in the upper cluster of the *preblei/campestris* clade. Is Custer, SD the most southern portion of the sampled range of *Z. h. campestris*? Could it be that the samples from Custer, SD represent instead the northern most part of the range of *Z. h. preblei?* I have no idea (it would be great to have more samples sequenced from that area), but it would be nice to see the haplotype data superimposed on a map so that one could investigate those questions.

Certainly the fact that no unique haplotypes are found in *Z. h. preblei* and that all four *Z. h. preblei* haplotypes are shared with *Z. h. campestris* is compelling evidence suggesting that *Z. h. preblei* and *Z. h. campestris* may be one in the same. It is interesting that the four *Z. h. preblei* haplotypes all group together which does suggests a founder event from *Z. h. campestris* with restricted gene flow. The authors use this evidence (along with morphological evidence) to conclude that, in fact, *Z. h. preblei* and *Z. h. campestris* **are** synonymous and even go so far as to suggest that *Z. h. preblei* does not qualify for protection under ESA as a DPS. One major problem with this conclusion is that the genetic data that they gathered is from only one locus (or one window of evolution). Further, this locus represents only the matrilineal history, which could very well differ from the evolutionary history of that species or subspecies. It has been shown in other rodent species that mtDNA patterns can be widely different than patterns in the nuclear genome due to introgression and (Prager et al. 1993, Ruedi et al. 1997). In fact, Ruedi et al. (1997) found that despite distinctive nuclear differences between subspecies of pocket gophers, mtDNA haplotypes were found to be very similar due to introgression. Thus, I would be very skeptical to conclude **undeniably** that *Z. h. preblei* and *Z. h.*

campestris are synonymous without including nuclear data. I would also like to see more data from each "population" of *Z. h. campestris*, particularly in the Custer, SD area. A population level study of *Z. h. preblei* and *Z. h. campestris* using nuclear and mitochondrial markers would do a better job of providing a definitive answer.

The authors claim that using all three criteria, they reject the idea that *Z. h. preblei* is a valid subspecies. Certainly the data show that *Z. h. preblei* are not reciprocally monophyletic. This concept, however, can be overly restrictive (in my opinion) and only utilizes genetic data, which in my mind, is problematic. The second criterion based on AMOVA has not been well tested (at least that I know about, see below) and therefore I am not comfortable using it to define a subspecies. The third criterion is conceptually a good one. It is based on comparing recent and historic exchangeability and is set up in a hypothesis-testing framework. Crandall et al. 2000 suggest that "individuals from different populations are genetically exchangeable if there is ample gene flow between populations" and by ample gene flow they suggest "unique alleles, low gene flow estimates ($Nm<1$) or phylogenetic divergence concordant with geographic barriers". The authors state that the populations are genetically exchangeable because of shared haplotypes and no unique alleles. It would be interesting to estimate levels of gene flow. My biggest problem with this criterion is how the authors report their finding of ecological exchangeability. They state that they found ecological exchangeability based on a review of the literature. They give no explanations of what variables were compared or even any citations of any of the literature that was reviewed. This gives me no avenue to repeat the analysis that they did or even to judge whether or not I think it is valid. The authors emphasize how their study is based on testable hypotheses and the scientific method. Therefore, I find it troublesome that they included this assessment of the literature and made strong conclusions without reporting any of the data or the citations.

Specific comments:

Pg 3 – It is unclear from this report whether the Preble's Meadow Jumping Mouse was listed as a DPS or subspecies.

Pg 3 – Include a range map. The description of the species range states that it extends from "the Pacific Coast of Alaska eastward to the Atlantic Coast; from the northern limit of tree growth south into central Colorado, Nebraska, eastern Kansas, Missouri, Tennessee, and northern Georgia" – What about the samples you obtained from New Mexico and Arizona?

Pg 4 – The authors state that they use population genetic methods that they only touch upon with AMOVA. I would like to see a real population level study comparing at least populations of *Z. h. preblei* and *Z. h. campestris.*

Pg 4 – I am skeptical of Ramey's method of defining subspecies based on the relationship between variability among vs. within populations. How does this method work across different molecular markers? Is it robust when comparing populations of different sizes? Is it robust to differences in sample sizes among groups? Further, I am not convinced

that it is always less restrictive than reciprocal monophyly.

Pg 13 – I would not state based on this data set alone that *Z. h. preblei* are not markedly separated from other populations. This data suggest that they may not be separate, but without further analysis I don't believe the question can be answered undeniably.
Pg 14 – I take issue with the fact that the authors state that for a mere 57,000 dollars (50,000 of which went toward genetic work) they have been able to redefine the taxonomic classification of *Zapus hudsonius*. While I do agree that they have made a good start to answering the question, without the addition of nuclear markers their data is severely limited. I believe that the cost of adding nuclear markers and additional samples to address the *preblei/campestris* question at a population level will not be trivial and that is misleading to USFWS and other agency personnel to suggest otherwise.

Sara Oyler-McCance's answers to specific questions to consider for review of Dr. R.R. Ramey's report on genetic analysis of Preble's Meadow Jumping Mouse

Please analyze the techniques used in the population and phylogenetic evaluation of *Zapus hudsonius preblei* and other taxa. Were appropriate methodologies and markers used?

The use of mitochondrial control region data is an appropriate marker to use to begin to address the taxonomic question at hand. It is important, however, to include nuclear markers as well before definitive answers about taxonomic delineations are made. The authors used the proper methodology for the phylogenetic analysis. I am less comfortable with the "population analysis" mostly because it is based solely on only one test, AMOVA, and the conclusions drawn by the authors regarding the ratio of between vs. within group variation are based on a metric that is largely untested (see comments regarding this elsewhere).

Are the conclusions about the taxonomic validity of *Z.h. preblei* logical and defensible as presented in the manuscript?

I have no problems with the study itself except for some of the conclusions made by the authors. I feel that in some cases they have made recommendations based on an incomplete data set. Their data may *suggest* that *Z. h. preblei* and *Z. h. campestris* are synonymous yet without collecting data from nuclear loci, I would not say definitively that they are and I feel it is wrong to suggest reclassifying *Z. h. preblei* without collecting nuclear data and doing a more complete population level analysis first.

Are there possible alternative interpretations of the genetics data? Are there additional or divergent taxonomic conclusions that could be drawn from the genetics data?

Other studies have found a discordance between mitochondrial and nuclear data sets. It is possible that nuclear data might reveal a difference between the two subspecies that

was masked in the mtDNA through introgression. Further, I would be interested in seeing more data from the Custer, SD sampling site. It seems a little odd to me that 5 of the 7 *Z. h. campetris* samples that most closely resemble *Z. h. preblei* all are found in one location and that there are no other *Z. h. campetris*-like samples in that sampling locale.

Do you agree with the interpretation about possible mechanisms of reduced gene flow between *Z.h. preblei* and other subspecies of *Z. hudsonius*?

I do agree with the authors that the data seem more consistent with a southward colonization from *Z. h. campestris*. Again, it would be really nice to have a figure showing haplotype frequencies superimposed on a map. It appears as though the connection between the two subspecies is through Custer, SD (are these samples misidentified?). Is this the closest population to the *Z. h. preblei* group? It would be interesting to have estimates of gene flow using coalescent theory. Due to the low haplotype diversity within *Z. h. preblei*, it seems reasonable that there is reduced gene flow (compared to *Z. h. campestris*). A population level analysis including populations from both subspecies could better answer that question.

Do you agree with the concepts of Crandall et al. (2000)* for defining evolutionarily significant units?

Conceptually I think the concepts of Crandall et al are reasonable. The nice thing about this concept is that it focuses on the importance of adaptive distinctiveness in populations and because it combines genetic and ecological data. The hypothesis testing aspects of it are less appealing to me because of the inherent problems with applying hypothesis testing to observational data. Additionally, I don't feel that the concepts of Crandall et al (2000) are necessarily any better than some of the other concepts that are in the literature.

Are there clear ecological distinctions between *Z. h. preblei* and closely related taxa that would suggest a need for specific conservation actions for this taxon?

I know nothing about the ecological distinctions between the subspecies and am concerned that the authors used this as a criterion ala Crandall et al. 2000 yet failed to report what variables they used or even cite the literature that they examined to make this assessment.

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March 20, 2004

Gary Skiba
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Dear Mr. Skiba:

This letter is to address your request for my comments on Dr. R. R. Ramey's report "Testing the taxonomic validity of Preble's Meadow Jumping Mouse (*Zapus hudsonius preblei*)". I am not an expert on genetic analyses, so am not able to comment on the specifics of the analyses presented in the report. Likewise, I am not an expert on morphometric analyses, so cannot comment on these measurements.

I will comment on the conclusions provided in the report based on the logic presented. Namely, this report concludes that there is no basis to distinguish between *Z. h. campestris* and *Z. h. preblei* because the authors did not find a difference with the tools they used. This conclusion is equivalent to stating that a Chevy 4-door wagen is equivalent to a Corvette because both use gasoline, both are shiny, both have windows, and both run on rubber tires. Both vehicles share many, many similar qualities, but are still very different vehicles.

The problem is that it is logically much easier to state that two items are different if the proper metric is measured. In contrast, one can never state that 2 items are identical, even if many, many measurements are taken, because the one critical difference between the two items was not measured or detected in the analysis. This report concludes that the two subspecies are the same, based on a limited suite of measurements. In reality, the report should conclude that no differences were detected given the measurements conducted, and should not jump to the unfounded conclusion that the two subspecies are identical. The conclusions presented in the report are much too strong given the necessarily limited set of measurements used.

Most importantly are the limited inferences that can be drawn from genetic measurements concerning important differences. Mitochondrial DNA sequence data could not distinguish a miniature dachshund from a Saint Bernard. Likewise mitochondrial DNA sequence data cannot distinguish a fall run salmon stock from a spring run salmon stock of the same species, even though this behavioral trait is critical to the survival of each stock. Wayne and Morin (2004) emphasize that the vast majority of conservation genetic evaluations are based on neutral markers which are in influenced by genetic drift. Quoting Wayne and Morin (2004:93-94):

> Specifically, neutral markers may often be poor surrogates for levels of variation in fitness traits (Reed and Frankham 2001). Furthermore, measures of population differentiation based on the analysis of quantitative traits, such as life history

> characteristics, may not be well correlated with measures based on neutral markers (McKay and Latta 2002; Merila and Crnokrak 2001). Conservation units based on historical isolation alone may not capture the adaptive variation necessary for populations to thrive in the short and long term, given changing environmental conditions (Crandall et al. 2000). Consequently, conservation genetic surveys should include neutral markers to assess population history and demography, as well as assays of fitness-related traits to preserve adaptive diversity.

Wayne and Morin (2004) provide further justification for why additional metrics than mitochondrial DNA and morphometric measurements are needed, such as analysis of natural history, functional aspects of the genotype and phenotype, and habitat data.

In summary, the conclusions in the Ramey et al. report are an example of a basic statistical misinterpretation. They were unable to reject the null hypothesis of no differences between *Z. h. campestris* and *Z. h. preblei* based on either genetic or morphometric procedures, so they concluded that the null hypothesis of no difference between *Z. h. campestris* and *Z. h. preblei* is true. As discussed above, such a conclusion is not supported by the data, and in fact can never be made with certainty.

Sincerely,

Gary C. White
Professor

Literature Cited

Wayne, R. K., and P. A. Morin. 2004. Conservation genetics in the new molecular age. Frontiers in Ecology and the Environment 2(2):89-97.

Literature Cited in quote from Wayne and Morin (2004)

Crandall, K. A., O. R. P. Bininda-Edmonds, G. M. Mace, and R. K. Wayne. 2000. Considering evolutionary processes in conservation biology. Trends in Ecology and Evolution 15:290-295.

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Merila, J., and P. Crnokrak. 2001. Comparison of genetic differentiation at marker loci and quantitative traits. Journal of Evolutionary Biology 14:892-903.

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