

Review of “Testing the uniqueness of *Z. h. intermedius* relative to *Z. h. campestris*” by Ramey et al. 2004

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In this report, the authors investigate the taxonomic relationships among five previously defined subspecies of *Zapus hudsonius* using both genetic and morphological data. Such taxonomic delineations, in my opinion, should always be investigated using multiple lines of evidence (typically genetics, morphology, behavior, and geography) so I am in agreement with the approach taken by Ramey et al. to address this question. I will focus my comments primarily on the molecular aspects of this work and less on the morphological aspects, as dictated by my knowledge and experience.

Previous reports by Ramey et al. have advocated that *Z. h. campestris* and *Z. h. preblei* should be considered one subspecies rather than two. This report investigates whether *Z. h. campestris*, *Z. h. preblei*, and *Z. h. intermedius* should all be considered one subspecies. Previously, I commented that I thought it was premature to synonymize *Z. h. campestris* and *Z. h. preblei* given several questions I had about the data set and given that Ramey et al. had considered only mitochondrial DNA and not nuclear markers. This report does not address the questions posed in my original review and does not include any data from nuclear markers. Therefore, I again recommend that it is premature to consider combining any of the previously described subspecies until certain aspects (discussed below) of the data set are resolved and before data from the nuclear genome are collected.

#### General Comments

In a previous report, the authors discuss the definition of a subspecies, specifically noting the definitions of Ball and Avise (1992), Moritz (1994), Crandall et al. (2000) and Ramey’s own definition that makes use of AMOVA. There is much discussion and disagreement in the scientific community regarding the definition of species and even more so regarding the definition of “conservation units” below the species level (e.g. subspecies, evolutionary significant units [ESU], or management units [MU]). While Moritz’s definition of an ESU (reciprocal monophyly) is a desirable one because it is easy to quantify and apply, it has been criticized for a variety of reasons (Waples 1995, Taylor 1999, Crandall et al. 2000, and Fraser and Bernatchez 2001) and was considered by Ramey et al. to be too restrictive of a definition in a previous report. Avise and Ball (1990) defined a subspecies as “groups of actually or potentially interbreeding populations phylogenetically distinguishable from, but reproductively compatible with, other such groups. Importantly, the evidence for phylogenetic distinction must normally come from the concordant distribution of multiple, independent genetically based traits.” A difficulty with this definition is quantifying what “phylogenetically distinguishable” exactly means. While this definition is mentioned by Ramey et al. it cannot be tested here because the authors collect data from only one genetic locus. The Crandall et al. (2000) definition also mentioned by the authors is, in my opinion, very difficult

logistically to assess given the amount of data required to test all the possible hypotheses (e.g. how can we be sure which ecological variables are biologically meaningful to the species in current time and how is it possible to measure them in the historical past?). In their previous report, the authors mention that “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* and *Z. h. campestris*” yet there is no discussion of what that literature actually showed (e.g. what variables were measured, etc) and no way to critically assess this important aspect of Crandall et al.’s (2000) definition. In this report, the authors base their conclusions entirely on Ramey’s own criterion that “there must be greater diversity among putative subspecies than within them”. This criterion, while discussed in three papers published by Ramey and colleagues, has not been tested in the literature or accepted by the scientific community as a standard. Ramey et al. state that this measure is less restrictive than Moritz’s (1994) standard (reciprocal monophyly) yet when *Z. h. preblei* is compared to *Z. h. intermedius*, *Z. h. preblei* would be considered an ESU according to Moritz (1994) but would not be considered a subspecies using Ramey’s AMOVA criterion. The amount of genetic diversity of a population or subspecies is dependent upon its demographic history. Ramey’s criterion is confounded by the sample size (how well a population or subspecies is characterized genetically), the resolution of the marker being used, and the amount of genetic diversity present (which again is a reflection of the history of the population or subspecies). In this report, Ramey et al. should have addressed the other subspecies concepts and how their data fit with those ideas of Avise and Ball (1990), Moritz (1994), and Crandall et al. (2000) (i.e. the concepts that have been discussed and scrutinized by the scientific community).

The molecular data in this report describes variation at a single mitochondrial locus among previously described subspecies. There has been extensive literature describing the potential problems of using mitochondrial sequence data alone to address population and taxonomic questions. 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. Additionally, this data set provides only a gene tree rather than a species tree, which would more accurately describe the phylogeographic history of the species and which should be used to make management decisions about the subspecies.

The new samples of *Z. h. intermedius* added to this report do show a close relationship between *Z. h. intermedius* and *Z. h. campestris* (in fact genetic distances between *Z. h. intermedius* and *Z. h. campestris* are closer than those between *Z. h. campestris* and *Z. h. preblei*). However, when *Z. h. intermedius* and *Z. h. preblei* are compared, they share no haplotypes and no gene flow, which makes the argument for combining them questionable even though they are bridged by *Z. h. campestris*.

I am still uncomfortable that a large part of the argument for combining *Z. h. campestris* and *Z. h. preblei* rests with five samples of *Z. h. campestris* from Custer, South Dakota and two samples of *Z. h. campestris* from Carter, Montana. From my understanding of the table in the report, all the individuals from Custer, South Dakota were identified as *Z. h. campestris* yet have haplotypes consistent with *Z. h. preblei*. In their first report, Ramey et al. dropped certain samples out of the study based on their genetic haplotypes assuming that they had been misidentified using morphological characteristics. I presume it is then possible that the five individuals from Custer, South Dakota could have been misidentified morphologically. I would have liked to compare the morphological measurements of those five individuals of *Z. h. campestris* from Custer, South Dakota with the morphological measurements from *Z. h. preblei* yet the voucher numbers in the report for the genetic data do not match up with the voucher numbers associated with the morphological data on the USFWS website, so again, there is no way to test this hypothesis. If those five samples from Custer, South Dakota were misidentified, it would have a significant impact on the data analysis and the determination of whether *Z. h. preblei* is a valid subspecies. A further frustration with this report is that the voucher numbers in Table 1 do not always match with the voucher names entered into Genbank.

#### Answers to Specific Questions Posed

- 1) Analyze the techniques used in the morphometric, the population and phylogenetic evaluation, and the maximum likelihood of recent gene flow analysis (MDIV) of subspecies of *Zapus hudsonius*. Were the appropriate methodologies and markers used? Do you support the authors' mtDNA standard for delineating valid subspecies (greater variation among subspecies than within subspecies)?

Regarding the molecular side of this study, the authors use an appropriate genetic marker (mtDNA sequence data) and do a good job analyzing the data from a phylogenetic standpoint. I feel that from a population standpoint, their study is weak due to small sample sizes, yet the goal of the study was not to study populations within a subspecies, but rather to investigate the taxonomic validity of the subspecies. Their report provides some good basic information regarding the relationship among the previously described subspecies, yet it by no means resolves the taxonomic questions. Further, I feel that it is a bit reckless of the authors to suggest otherwise. I question the seemingly haphazard way that some samples were dropped from the original report because they were assumed to be misidentified. In this report, the authors use MDIV to estimate levels of gene flow among previously described subspecies and use that as a measure of connectedness. While I believe that the methodology and the software are appropriate, I am concerned about how the authors used it and the conclusions that they drew from it. First, an assumption of the program is that all population sizes are equal (see URL <http://www.binf.ku.dk/users/rasmus/webpage/programs.html>). I am concerned that this assumption is likely violated here particularly with the vastly different  $N_e$  reported among pairs of subspecies. There is no discussion in this report about the potential effect on the estimators if this assumption is violated. Second, the authors estimate a mutation rate for their 346 bp of control region mtDNA sequence data using published sequence

divergence from five species of Red-Backed Voles (Matson and Baker 2001). They then use this mutation rate to solve for  $m$  and  $M$ . Mutation rates are highly variable across different parts of the control region. The authors provide no description of how they calculated the mutation rate that they used ( $2.5 \times 10^{-5}$ ) and it is not obvious from reading the paper by Matson and Baker (2001). Further, there is not even any description in any of the reports by Ramey et al. about which section of the control region was sequenced. Thus, it is impossible to determine whether the calculations that the authors used for mutation rates are reasonable or not (even though the estimates of  $m$  and  $M$  are highly dependent on them). Additionally, there is no estimate of uncertainty around that mutation rate. Given these concerns about how the estimates of  $m$  and  $M$  were determined by the authors, I have little confidence in the actual estimates. Assuming that I was confident in those estimates, however, my interpretation of a subspecies is similar to that of Avise and Ball (1990), which allows some genetic interchange among subspecies. It is my feeling that subspecies are not necessarily reproductively isolated and that individuals of different subspecies can and will interbreed. To me, the fact that there may be small levels of gene flow among previously described subspecies does not negate their status as valid subspecies. Finally, I do not support the authors' mtDNA standard for delineating valid subspecies. This standard has not been accepted or even discussed by the scientific community. The authors suggest that it is a less restrictive definition than reciprocal monophyly and I do not believe that it is in all cases (see specific case discussed in general comments). Additionally, I believe that because so many different factors affect the amount of measured genetic diversity in populations or subspecies (such as the resolution of the markers used in the study, the sampling scheme and sample size, and the demographic history of the populations) this method will not provide consistent results across similar sets of data and is confounded by such factors.

- 2) Based on the data presented in the report do you support the authors' conclusions regarding synonymizing *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius*?

The data presented in this report provides only general information regarding the relationship among the previously described subspecies. I do not support the authors' conclusion to synonymize *Z. h. preblei*, *Z. h. campestris*, and *Z. h. intermedius* based on this data set. I feel it is premature to even consider changing the taxonomic status of these subspecies without first examining data from the nuclear genome and also addressing the specific issues stated above.

- 3) Based on the MDIV data presented in the report, do you view *Z. h. preblei* and *Z. h. campestris* as a single connected population?

There are several issues surrounding the MDIV data that make me question the results and the conclusions of this analysis. First, MDIV assumes that the populations being compared are of equal size. It is unclear what the effect of violating that assumption may be. It seems troubling to me, however, that the calculated  $N_e$  values are so different for all pairs of populations (ranging from 27,409 – 230,924). This raises a red flag about violating the assumption of equal population sizes. Second, a major variable in the

equation is the mutation rate of the mitochondrial region being compared. The authors do some calculations based on published mutation rates across the entire mitochondrial control region of several species of Red-Backed Vole and apply that mutation rate to their data. They do not provide any details at all of how they have calculated this rate and it is **vital** to the estimates of  $m$  and  $M$ . Thus, there is no way to determine if their mutation rate calculation is valid or not. Even if I could be convinced that the differing population sizes did not matter and that their use of mutation rate was valid, I would still be hesitant to consider *Z. h. campestris* and *Z. h. preblei* a single connected population with migration rates on the order of one individual per generation (I assume that the units for  $M$  are number of migrants per generation, yet the authors never state what the units are).

- 4) Are there possible alternative interpretations of the data? How likely are these possibilities?

An alternative interpretation for the data is that *Z. h. campestris* and *Z. h. preblei* are indeed separate entities and that there has not been sufficient time for complete lineage sorting. A point that seems to keep being missed here is that the four haplotypes found in *Z. h. preblei* are very closely related (form their own clade) and that their frequencies are vastly different than the frequencies of those same haplotypes in *Z. h. campestris* suggesting very little or no gene flow between those two groups. Further, it is possible that two of those haplotypes really are unique to *Z. h. preblei* if it turns out that the samples in Custer, SD have been misidentified.

- 5) What additional analysis, if any, is needed to verify the study's assertions and why?

The addition of nuclear markers is essential before any real conclusions can be made regarding these subspecies for all the reasons stated above. The completion of a nested clade analysis would provide valuable insight into documenting historic vs. recent events and would allow for the investigation of founder events. It would also be valuable to do a real population level analysis yet that would require greater sample sizes characterizing populations within a subspecies. Finally, I would like to see additional samples collected from the Custer, SD area and verification that they are in fact *Z. h. campestris* and not *Z. h. preblei*.

- 6) Has this new information changed your conclusions regarding the synonymizing of *Z. h. preblei* and *Z. h. campestris* as proposed in Ramey et al. 2004a? Please elaborate as necessary.

The new information presented in this report has not changed my conclusions regarding the synonymizing of *Z. h. campestris* and *Z. h. preblei*. There were numerous interesting and valuable comments provided by the reviewers of the first report, most of which were not addressed here. This report presents only additional data from one additional subspecies and does not address any of my concerns from the first review.

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