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Mistaken view of taxonomic validity undermines conservation of an evolutionarily distinct mouse: a response to Ramey *et al*. (2005)

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In a study self-defined in its introductory paragraph as an effort to roll back US Endangered Species Act (US-ESA) protection for a geographically isolated and currently recognized subspecies, in order to avoid misallocating financial and logistical resources, Ramey *et al*. (2005; hereafter REA) proposed to synonymize the threatened Preble's meadow jumping mouse *Zapus hudsonius preblei* with two currently unlisted subspecies, the prairie jumping mouse *Zapus hudsonius intermedius* and the Bear Lodge meadow jumping mouse *Zapus hudsonius campestris*. They stated a priori that their intention was to reach a conclusion that would be 'in the best interest of biodiversity conservation' and they subsequently argued that the data they presented in support of their recommended synonymy were cast in a light of unbiased hypothesis testing (REA). Despite these stated claims, they dismissed the geographic isolation of this population as unimportant, ignored most of the diagnostic characters initially cited in the taxon's original description by Krutzsch (1954), concluded without data or citation a lack of ecological distinctiveness of this population, and finally misinterpreted the morphological and molecular data they presented.

Zapus hudsonius preblei is currently a recognized taxon and a legally protected subspecies; thus, we regard its geographic and genetic isolation, occurrence in an ecoregion distinct from that of conspecifics (Chapman *et al*., 2004), and formally described distinctive phenotypes of pelage and skull shape (Krutzsch, 1954) as operative hypotheses that must be explicitly disproven for synonymy to be accepted. REA proposed synonymy of *Z. h. preblei* based on four main lines of evidence – ecological differentiation, cranial morphology and analyses of mitochondrial DNA and nuclear microsatellites – and implied that their study should serve as a model of a 'conceptually sound and consistent methodological approach' for evaluating the genetic basis for listing under the US-ESA. We find that despite the potential for objective interpretation, REA reached conclusions that were neither justified by the narrow scope of their study nor supported by the data they presented. Instead, we argue that their own data support the current classification of *Z. h. preblei* as a separate evolutionary unit and a genetically distinguishable subspecies.

It is impossible to predict future patterns of speciation; thus, in our efforts to preserve biodiversity, we must seek to maximize evolutionary potential through the protection of populations on separate evolutionary trajectories (O'Brien & Mayr, 1991; Hey *et al*., 2003). Given that the most important aspect of preserving biodiversity is protecting evolutionary potential, we are concerned that the erroneous application and interpretation of morphometric, genetic and ecological information presented by REA in an effort to subsume an evolutionarily distinctive population will not only undermine efforts to conserve this taxon but also serve as a misleading precedent applied to broader conservation programs.

Ecological analysis

REA dismissed the isolation of *Z. h. preblei* from conspecific populations, particularly *Z. h. campestris*, as merely 160 km; yet, this 160-km swath of non-habitat separating the

northern Front Range foothills from the Black Hills foothills is the widest separation between any two subspecies of *Zapus hudsonius* (Cryan, 2004) and as such constitutes a sufficient 'primary isolating mechanism' to stop or significantly reduce gene flow, a necessary criterion for the definition of a subspecies (Whitaker, 1970; O'brien & Mayr, 1991). Additionally, it has previously been established that substantial environmental differences occur between the ranges of each of these subspecies: *Z. h. preblei* is restricted to the grama–buffalo grass association, whereas *Z. h. campestris* is found in wheatgrass–needlegrass or grama–needlegrass–wheatgrass associations (Küchler, 1970). The inarguably different environments of these disjunct populations (Chapman *et al.*, 2004; Cryan, 2004) make it likely that, in the absence of significant gene flow, ecological phenotype has diverged between them. Given this geographic and environmental separation, we argue that the potential for ecological differentiation among these populations is high.

REA ignored this most conservative expectation and assumed that a lack of studies to test specifically for ecological differentiation among subspecies is equivalent to an actual lack of ecological differentiation. Further, while REA (pp. 330–331, 339–340) represented their ecological analysis as a 'method' with 'results,' they presented nothing that could be interpreted as a 'test' of 'ecological exchangeability.' REA claimed to have 'examined the literature' for evidence of ecological differences between subspecies, but they neither provided detailed methods for the selection and evaluation of articles nor supported their assertion with *any* type of statistical analysis. REA admit their 'absence of evidence' is not 'evidence of absence'; their conclusion of 'ecological exchangeability' is an unsupported opinion.

A search covering 1965–2005 on the ISI Web of Knowledge (http://portal17isiknowledge.com) produced only six studies (Bain & Shenk, 2002; Schorr & Davies, 2002; Brook, Zint & De Young, 2003; Conner & Shenk, 2003; Meaney *et al.*, 2003; Ramey *et al.*, 2005), including REA, for '*Zapus hudsonius preblei*' or 'Preble's meadow jumping mouse,' none of which tested ecological phenotype, and no studies for '*Zapus hudsonius campestris*,' '*Zapus hudsonius intermedius*' or their respective common names. Clearly, the question of ecological exchangeability among these subspecies simply has not been posed. The lack of peer-reviewed publications on the ecology of *Z. hudsonius* subspecies (e.g. life-history characteristics, population dynamics and viability, and habitat selection; Cryan, 2004) indicates that solid research on these populations is needed before *any* conclusions can be reached about their ecological distinctiveness or exchangeability. We reject REA's claim that they conducted a test for ecological exchangeability and stress that until the question of ecological exchangeability is investigated directly, this line of inquiry is uninformative as to the question of divergence among these taxa.

Morphometric analysis

Krutzsch (1954) described 11 characters that distinguished the disjunct population of *Z. hudsonius* along the Colorado (CO) and Wyoming (WY) Front Ranges from its most similar conspecific, *Z. h. campestris* of the Black Hills–Missouri Plateau; five of these were qualitative descriptions of pelage and six were skull characteristics. The six skull characters included interorbital breadth, size and shape of auditory bullae, width and shape of incisive foramina, and degree of inflation of the frontal region. REA examined none of the pelage characters, and of the nine cranial measurements REA examined, only one – interorbital breadth – was among the six cranial characters actually cited by Krutzsch as distinguishing *Z. h. preblei* from *Z. h. campestris*. Of the cranial metrics REA used, five included greatest length of skull (GLS) or measures highly correlated with GLS, and the other four were measures of skull breadth. Interestingly, of the 36 pairwise Pearson correlation coefficients among these nine variables, 26 were significant at $P<0.001$ (two-tailed α; Minitab, 1996; raw data from U.S. Fish and Wildlife Service (USFWS, 2004).

No univariate or multivariate analysis of these metrics could possibly have resolved the incisive foramina, auditory bullae or frontal inflation size/shape characters cited by Krutzsch (1954) as constituting 'considerable differences.' Therefore, REA have conducted an incomplete test of the morphologic hypothesis put forth by Krutzsch. Importantly, the sole univariate character cited by Krutzsch that REA did examine, interorbital breadth, was found to be narrower in *Z. h. preblei* than in *Z. h. campestris*, as described in the definitive findings (Krutzsch, 1954). Thus, the small fraction of Krutzsch's morpho-taxonomic hypothesis actually tested by REA confirmed Krutzsch's initial findings of distinctiveness for *Z. h. preblei*. Oddly, their conclusions imply the opposite. REA apparently viewed a multivariate statistical test of a standard set of morphologic variables, although incomplete and intercorrelated, as a substitute for attempting to quantify the specific shape differences noted by a trained morpho-taxonomist. One should not expect such an arbitrary, hypothesis-free approach to resolve subspecies relationships (Gift & Stevens, 1997; Poe & Wiens, 2000); examples of the failure of this blind approach abound, even when comparing full species (e.g. Poole, Carpenter & Simms, 1980; Zink 1988; e.g. Barratt *et al.*, 1997).

Molecular genetic analyses

Mitochondrial DNA

Although mtDNA is still occasionally used as the sole locus in phylogenetic studies, it is accepted that if doing so, sequence length should be maximized as any single locus will be subject to variation of *d*, the number of substitutions per site, and this variation will be reduced as the number of sites sequenced per gene is increased (Arbogast *et al.*, 2002). A much more accepted and accurate approach for obtaining a gene genealogy (gene tree) reflective of the true lineage genealogy ('species' tree), however, is the inclusion of multiple independent loci (Edwards & Beerli, 2000). The examination of divergence patterns across multiple loci decreases

the coalescent variation (the stochastic variance in gene divergence times which arises due to genetic drift; Arbogast *et al*., 2002) and thus vastly improves the estimate of the true history of a lineage. When only a single locus is used to construct a phylogeny, discordances between this single locus gene tree and the actual species tree will be expected due to ancestral polymorphism and incomplete lineage sorting (Maddison, 1997; Arbogast *et al*., 2002). These processes are expected to be even more pronounced in recently diverged lineages and those with structured populations (Wakeley, 2000, 2001), as would be expected in this habitat-specific subspecies group. Despite these well-understood expectations, REA used only a single, short [346 base pairs (bp)] region of the mtDNA control region to test for divergence among the *Z. hudsonius* subspecies group and then treated the patterns of divergence observed within this single region as equivalent to the patterns of divergence among the subspecies.

We caution that the mtDNA data presented by REA should be viewed as preliminary. However, we find that in their current state they are nonetheless consistent with the expectation of incomplete lineage sorting and are indicative of divergence among the subspecies examined. Although bootstrap support for the split between the *Zapus hudsonius luteus*/*Zapus hudsonius pallidus* and *Z. h. preblei*/*Z. h. campestris*/*Z. h. intermedius* clades was high, support was quite low for REA's terminal clades ($\leq$ 50–68%); thus terminal branching patterns within this phylogeny should be considered hypotheses with little support (we note in particular that terminal branch support for clades that grouped *Z. h. preblei* with *Z .h. campestris* appeared to receive support of $<$52%). Nonetheless, all individuals identified a priori as *Z. h. preblei* grouped within a single clade. REA put forth reciprocal monophyly (Moritz, 1994*b*) as the sole criteria for accepting divergence among subspecies; however, given the expectation of incomplete lineage sorting, this requirement was overly stringent, and it being the sole criteria for acceptance of divergence increased the likelihood that REA would conclude that no differences exist among subspecies. Notably, and consistent with an understanding that incomplete lineage sorting can complicate the understanding of phylogenetic history, Moritz (1994*a*) modified his proposal of reciprocal monophyly with the suggestion that significant, but not necessarily absolute, separation of alleles among populations is an appropriate indicator of the presence of distinct, taxonomically recognizable entities.

Although we find the current phylogeny generated by REA to be preliminary, the marked differences in haplotype frequencies observed among the five subspecies clearly support divergence. In order to further explore the pattern of haplotype frequencies among the different subspecies, we designated each observed haplotype (from REA) to the subspecies within which it occurred with the highest frequency (calculated from Appendix 2 of REA); for example, all L and L/PAL haplotypes were assigned as '*luteus* haplotypes' (with frequencies in *Z. h. luteus* of 1.00), although they also occur in *Z. h. pallidus* and *Z. h. campestris* at much lower frequencies (0.059 and 0.129, respectively; Table 1). 'Contaminant' haplotypes may result from incomplete lineage sorting, migration from adjacent subspecies or misidentification of individuals at subspecific boundaries. Although both incomplete lineage sorting and migration of individuals from adjacent subspecies would be expected, other cases of supposed 'contamination' more likely result from misidentification of individuals. For example (Appendix 2 of REA), three individuals of '*Z. h. intermedius*' from Harding Co. in north-western South Dakota (Fig. 1) with the C5/INT13 haplotype (designated as a '*campestris* haplotype') are mapped by REA (their fig. 4) as occurring within the range of *Z. h. campestris*, and two individuals of '*Z. h. pallidus*' from Clay Co. in extreme south-eastern South Dakota (Fig. 1) with the PAL1/INT15 haplotype (designated as an '*intermedius* haplotype') are the only '*Z. h. pallidus*' found within the range of *Z. h. intermedius,* north of the Missouri River. Even if we assume these individuals were correctly assigned to subspecies, *Z. h. preblei, Z. h. luteus, Z. h. intermedius* and *Z. h. pallidus* exhibited low frequencies of 'contaminant' haplotypes of all types, whereas *Z. h. campestris* contained an admixture of haplotypes (Table 1, Fig. 1).

Table 1 Frequency of subspecies characteristic haplotypes (assigned to subspecies based on highest frequency of occurrence) within five subspecies of *Zapus hudsonius*

	Subspecies				
Haplotype	*Preblei*	*Luteus*	*Intermedius*	*Pallidus*	*Campestris*
Preblei	**1.000**				0.226
Luteus		**1.000**		0.059	0.129
Intermedius			**0.915**	0.059	0.258
Pallidus			0.021	**0.882**	
Campestris			0.064		**0.387**

The frequency at which each subspecific haplotype is found within each subspecies is shown in boldface along the diagonal; squares indicate ancestral haplotypes shared likely due to incomplete lineage sorting; ovals indicate results of possible migration or mistaken subspecific identification (based on geographic location).

The unique admixture of haplotypes in *Z. h. campestris* may indicate a previously more widespread distribution (allowing retention of ancestral haplotypes), may simply reflect that subspecies' geographic position adjacent to three other subspecies (opportunities for migration and misidentification), or a combination of both factors. Notably, no contaminant haplotypes were found in *Z. h. preblei,* and although '*preblei* haplotypes' were also found in the highly admixed *Z. h. campestris,* the haplotype frequency differences between these subspecies were striking (Fig 1, Table 1 and REA fig. 3). This pattern of significant haplotype frequency differences occurring in conjunction with a lack of reciprocal monophyly for two closely related lineages is consistent with the process of incomplete lineage sorting wherein ancestral polymorphism of haplotypes is retained across divergent lineages at low frequencies (Avise, 2000). Such incomplete sorting of haplotypes is not only expected theoretically, but has also been well documented in a wide

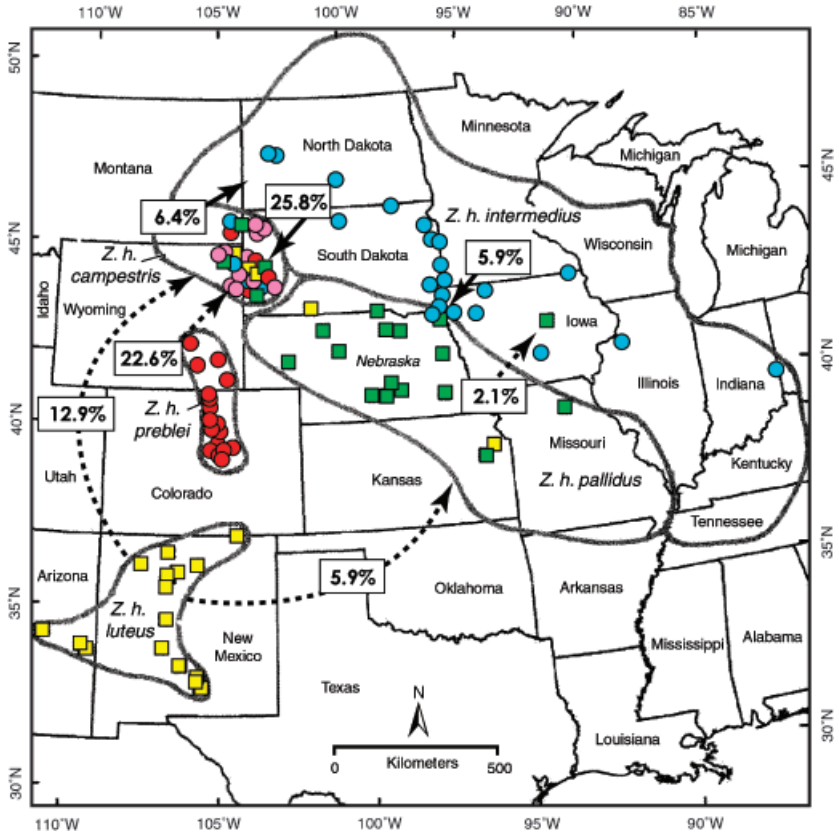

Figure 1 Distribution of mtDNA haplotypes among five subspecies of *Zapus hudsonius*. Squares = *pallidus-luteus* lineage, circles = *intermedius-campestris-preblei* lineage (from fig. 3 of Ramey *et al.* 2005). Colors (modified from the original figure) indicate haplotype assignment to subspecies (see text and Table 1). Percentages of haplotypes characteristic of one subspecies and found in another are indicated within boxes next to arrows. Solid arrows indicate probable migration or mistaken subspecific identification of samples; dotted arrrows indicate probable shared-ancestral haplotypes due to incomplete lineage sorting.

variety of organisms, including taxa that are clearly separate biological species (Avise, 2000).

Given the availability of rapid DNA sequencing technology, universal primers for mtDNA amplification and numerous nuclear loci for mammals (48 reported by Yang & Nielsen, 1998), the short sequence of the single mtDNA locus used by REA represents a minimal effort toward revealing patterns of divergence in this group and should be observed as only a preliminary foray into its true evolutionary history. Many studies investigating similar questions of lineage divergence have used much higher standards and these should be viewed as more solid models for taxonomic investigation. For example, Roca *et al.* (2001) used 1732 bp from four nuclear DNA genes to separate African forest elephants from savannah elephants as separate species. Culver *et al.* (2000) used 891 bp of mitochondrial DNA and 10 DNA microsatellites to collapse 15 historically recognized subspecies of puma into six subspecies, and Jones *et al.* (in press) used 1900 bp of combined mitochondrial and nuclear DNA sequences and 10 DNA microsatellites to distinguish populations of endangered freshwater mussels as either species or subspecies. These studies also used geography, life history, behavior and morphology to corroborate their findings. Given the strength of the arguments for the use of multiple loci in phylogenetic studies and the prevalence of numerous studies demonstrating much higher standards of data inclusion, the single-locus, short sequence approach used by REA should be viewed as precursory and most certainly should not be presented as an adequate basis for the making of taxonomic decisions regarding a listed taxon.

The taxa investigated by REA clearly violate an assumption of the MDIV test for gene flow among subspecies, the assumption of equal effective population size (N_e). Nevertheless, if we assume their estimates are generally accurate, the degree of gene flow between *Z. h. preblei* and *Z. h. campestris* is very low, an unscaled rate of 0.000033 to 0.0000032 individuals per generation. This rate does not qualify as homogenizing gene flow. Natural hybridization among well-differentiated species can occur at rates higher than this (e.g. Campton & Utter, 1985; Arnold, 1992; Roques, Sevigny & Bernatchez, 2001), and low levels of gene flow do not preclude local adaptation (Broggi *et al.*, 2005). Although complete introgressive hybridization

(i.e. hybrid swarms) may exclude hybridized populations from the units considered for listing under the US-ESA (Allendorf *et al.*, 2004), REA quite clearly demonstrate that this level of introgression is *not* occurring among *Z. h. preblei* and other subspecies.

Microsatellites

Similar to the analysis of mtDNA sequence data, REA used too few loci in the microsatellite analysis to ensure high resolution. Smouse & Chevillon (1998) state that 'large numbers of polymorphic loci' are required 'to assign individuals to their correct population' and emphasize that there is a positive relationship between the number of populations in question and the number of loci required to place individuals correctly. In initially describing the STRUCTURE method used by REA, Pritchard, Stephens & Donnelly (2000) were unable to acquire a clear estimate for K (the number of populations represented within the sample) with their simulated dataset using five polymorphic loci. Further, they concluded that 'the accuracy of assignment depends on. . .the number of loci [which will affect the accuracy of q_{MAX} (likelihood of assignment of an individual to a given cluster)].' Although locus availability is often a problem, as of 2003 there were at least eight additional microsatellite loci for *Zapus* spp. (Vignieri, 2003) available for use by REA.

Given the expected low resolving power of the microsatellite data, REA's results are surprisingly strong in support of differentiation of *Z. h. preblei* from the other subspecies. F_{ST} values that are significantly different from zero indicate that gene flow among the compared populations is limited enough to result in genetic divergence. F_{ST} values observed among *Z. hudsonius* subspecies were significant for all pairwise comparisons, indicating that variation in allele frequencies among subspecies was greater than that within subspecies (Wright, 1951; Weir & Cockerham, 1984); thus the subspecies are genetically diverged. Although REA argue that their observed F_{ST} values are low (0.07–0.16), they are well within the range generally observed among subspecies in mammals (gray wolf 0.168, Roy *et al.*, 1994; African buffalo 0.059, Van Hooft, Groen & Prins, 2000; jaguar 0.065, Eizirik *et al.*, 2001). Further, REA report high per-locus polymorphism and high values of within-population heterozygosity, H_s (0.69–0.94). Considering the value of F_{ST} can be no larger than $1-H_s$ (Hedrick, 1999), even with complete differentiation, the highest absolute F_{ST} we would expect for the loci used by REA ranges from 0.06 to 0.31, and thus the F_{ST} values observed among subspecies are relatively high.

Strong support for differentiation among subspecies is also found in the STRUCTURE analysis. Although resolving power with five loci is limited, q_{MAX} for both *Z. h. preblei* populations was quite high ($q_{MAX} = 0.85$ for the northern population and 0.86 for the southern population). All other subspecies had lower q_{MAX} values, including *Z. h. luteus* (0.67), whose distinctiveness REA do not question. Similarly, correct assignment proportions for both northern (42.9%) and southern (54.5%) populations of *Z. h. preblei* were considerably higher than those observed in any other subspecies, including *Z. h. luteus* (only 21.9% of individuals correctly assigned). Additionally, 95% of the northern population and 94% of the southern population of *Z. h. preblei* were assigned to two clusters (2 and 5) that had very few individuals assigned from any of the other subspecies (REA table 6). Given the low resolving power of the loci used by REA, the relatively high proportion of correct assignment observed in *Z. h. preblei* populations provides further strong evidence of differentiation.

Use and Interpretation of AMOVA

REA used AMOVA as a measure of distinctiveness of *Z. h. preblei*, and set the criterion that there must 'be greater molecular variance among than within subspecies.' Results from mtDNA sequences showed that 18.5–37% of variation was found between subspecies, and microsatellite data indicate that 7.5–9% of variation occurred between populations. Although the authors do not present a significance value for the AMOVA test, they claim that *Z. h. preblei* fails these tests of genetic uniqueness. However, the within-population component of total genetic diversity may exceed the between-population component even when comparing separate species. For example, Leibers, Helbig & De Knijff (2001; using mtDNA sequence data) found that only 26.8% of the total diversity among gull populations resides among acknowledged species. Using microsatellite data, Grobler *et al.* (2005) found that only 29.2% of the total variation among blue and black wildebeest populations occurs between species. Thus, it is not necessarily expected that an AMOVA-based analysis of subspecies, or even species, will reveal more diversity among than within subspecies. The criterion used by REA was dubious at best, and the conclusion drawn from failure to meet this criterion is not valid.

Conclusion

The definition of taxonomic groups has long been an area of contention. Species concepts are abundant and continuously debated (a recent count listed 24; Mayden, 1997), and concepts of subspecies are even less well defined. Given the uncertainty present in both the definition of taxonomic status and the identification of such, in our efforts to preserve biodiversity we should be striving to protect populations of organisms that are on separate evolutionary trajectories rather than debating taxonomic definitions (Waples, 1991; Hey *et al.*, 2003). This desire has been expressed in both the literature and the intent of government policy where evolutionarily significant units (ESUs) and distinctive population segments (DPSs) have been identified as groups worthy of protection. Within the US-ESA, species are defined as 'any subspecies of fish or wildlife or plants, and any *distinct population segment* of any species of vertebrate fish or wildlife which interbreeds when mature' [16 U.S.C., Sec. 1532(16)] and it states that the definition of such groups should be determined based 'solely on the best

available science.' Clearly, the intent of conservation policy is to protect populations identified, in a scientifically rigorous way, as evolutionarily distinctive. Given the clarity of this intention, we find REA's recommendation of synonymy of *Z. h. preblei* curious and unjustified.

We firmly believe that no single approach should be used as a 'taxonomic litmus test' for taxa of conservation concern. However, for cases where such testing is appropriate, we offer a simple alternative hypothesis-testing approach based on the understanding that conservation of biodiversity requires conservation of groups that are evolutionarily distinct. Given this goal, we can address questions of conservation units based on this null hypothesis: These populations of individuals represent a readily interbreeding, undifferentiated unit with shared adaptations and a common evolutionary trajectory. What we are truly interested in revealing is whether there is *any* evidence that a given group is evolutionarily unique and therefore an important component of global biodiversity. Considering the data on *Z. hudsonius* subspecies presented by REA and other published information on the taxa and their environments we have discussed, we find the null hypothesis, that this group represents one readily interbreeding, undifferentiated unit, can be rejected, and the alternate hypothesis, that the populations currently classified as subspecies represent unique evolutionary entities, can be accepted across *all* of REA's informative lines of evidence. Gene flow between these disjunct subspecies is exceedingly low; there is evidence that *Z. h. preblei* is diverged in morphology and strong evidence that it is substantially diverged in mtDNA haplotype frequencies and microsatellite allele frequencies and allelic distribution.

Because REA assert a challenge to the Preble's meadow jumping mouse's current classification as a subspecies, the burden of proof is upon them to provide clear, solid evidence that this taxon is not evolutionarily distinct and thus its subspecific classification is unwarranted. Contrary to REA's stated conclusions, we find no evidence supporting their extreme recommendation of synonymy and instead conclude that their evidence offers further support for the classification of *Z. h. preblei* as a unique subspecies and a distinct evolutionary unit worthy of the protection it is currently afforded. Finally, we caution that vague questions of 'taxonomic validity' can undermine the intent to protect evolutionarily distinct units and we urge that this study not be considered a precedent for evaluation of validity in taxa of conservation concern.

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