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Misinterpretation of genomic data matters for endangered species listing: The subspecific status of the Peñasco least chipmunk (*Neotamias minimus atristriatus*)

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Abstract.—Puckett et al. (2021. *Ecology and Evolution, 11,* 12114-12128) evaluated the subspecies status of the Peñasco least chipmunk (*Neotamias minimus atristriatus*) using genomic approaches and concluded that their results did not support the distinction of this taxon as a subspecies and recommended it be synonymized with *N. m. operarius*. We refute the interpretations, conclusions, and taxonomic recommendations of Puckett et al. (2021), and explain in clearer terms how to interpret genomic analyses for applied management. We identify six conceptual issues that led to misinterpretations and errant recommendations: 1) error in hypothesis testing, 2) overlooking statistical support (or lack thereof) of lineages, 3) inappropriate use of reciprocal monophyly as a criterion for subspecies, 4) importance of geographic isolation and inferences from historical biogeography, 5) diagnosable criteria, and 6) importance of phenotype. We conclude that the data of Puckett et al. (2021) add to information from prior studies providing strong support for recognition of *N. m. atristriatus* as a subspecies. This finding has important and immediate implications for the proposed listing of *N. m. atristriatus* as an endangered species under the U.S. Endangered Species Act.

Keywords: conservation genomics, Distinct Population Segment, Endangered Species Act, independent evolutionary trajectory, integrative taxonomy, reciprocal monophyly

Recent decades have seen a surge in technological development of genomic sequencing methods for non-model organisms, along with associated bioinformatic data processing, and downstream evolutionary analyses. In parallel with these advances, genomic data are increasingly being applied to questions of taxonomic validity among wildlife, and towards systematic rearrangements within and among species of conservation concern. These applications may have consequential repercussions for regulatory legislation. It is therefore increasingly critical to bridge the “conservation genomics gap” for more effective exchange of knowledge among genomic researchers, conservation managers, and public stakeholders (Shafer et al. 2015; Taylor et al. 2017; Kadykalo et al. 2020). This will necessitate an improvement in how scientists translate technical jargon for knowledge-users, and will ensure that the scientific interpretation of results accurately reflects the limitations of the genomic data or analyses. As an example of these persistent issues, we discuss the results, interpretation and conservation-related recommendations from a recently published phylogenomic study of chipmunks, which bear on a pending decision of U.S. federal protections under the Endangered Species Act (ESA; Puckett et al. 2021).

Least chipmunks (*Neotamias minimus*) are the most widespread species within a diverse mammalian genus collectively distributed throughout western North America (Piaggio and Spicer 2001; Reid et al. 2012). There are currently 21 subspecies of least chipmunks that reflect a complex history of differentiation and diverse ecological and biogeographical associations (Verts and Carraway 2001). The main focus of Puckett et al. (2021) was to evaluate the taxonomic validity of the Peñasco least chipmunk (*N. m. atristriatus*), a geographically isolated subspecies at the southernmost extent of the species' range, and which has been extirpated from most of its historical distribution (Hope and Frey 2000; Frey and Boykin 2007; McKibben and Frey 2020). The taxon was described as a species by Vernon Bailey, a seminal mammalian taxonomist and naturalist, on basis of cranial and pelage characteristics (Bailey 1913). Bailey stated of *Eutamias atristriatus* (= *N. m. atristriatus*), that "In cranial characters this chipmunk shows so little similarity to *E. operarius* (= *N. m. operarius*), its apparently nearest relative, that I have given it full specific rank. A thorough revision of the genus may show some other species to which it is more nearly related, but its range is widely separated from that of any other small species" (Bailey 1913:130). This taxon is currently listed as Endangered within the State of New Mexico (NMDGF 2016) and has been proposed to be listed as endangered under the ESA at the taxonomic level of subspecies (USFWS 2021).

Puckett et al. (2021) performed a series of genomic data analyses of target-captured nuclear exons (DNA that codes for gene functions and which may or may not be subject to selection Luikart et al. 2018). They reported their data as 513 single nucleotide polymorphisms (SNPs) or 259 concatenated exon sequence loci, depending on the analysis. Their analyses also considered a mitochondrial genome dataset (maternally inherited haploid DNA). Taxonomic coverage included individuals representing 6 to 12 of the 21 recognized subspecies of least chipmunks (depending on analysis), as well as samples from several other species of chipmunk occurring in the southwestern U.S. Puckett et al. (2021:9-10) stated that their "… genetic data do not support the current distinct subspecies designation for *N. m. atristriatus*." From this conclusion they made the taxonomic recommendation to synonymize *N. m. atristriatus* with two other subspecies (*N. m. caryi* and *N. m. operarius*), for which *N. m. operarius* has nomenclatural seniority. Their interpretations of results that led to this recommendation included: 1) "…the clustering analyses, nuclear phylogenomic tree, and mitogenome haplotype network *unequivocally* grouped *N. m. atristriatus* with *N. m operarius* and *N. m. caryi* in the southern clade"; and 2) "Neither mitochondrial nor nuclear datasets identified *reciprocally monophyletic* diversity between *N. m. atristriatus* and the geographically proximate *N. m. operarius* and *N. m. caryi*" (Puckett et al. 2021:10; 13; emphases added by us). We refute the interpretation, principal conclusions, and taxonomic recommendations of Puckett et al. (2021; as outlined above). In this paper we identify six conceptual issues that led to these faulty interpretations and recommendations: 1) error in hypothesis testing, 2) overlooking statistical support (or lack thereof) of lineages, 3) inappropriate use of reciprocal monophyly as a criterion for subspecies, 4) importance of geographic isolation and inferences from historical biogeography, 5) diagnosable criteria, and 6) importance of phenotype. We conclude that the data generated by Puckett et al. (2021) support recognition of *N. m. atristriatus* at the subspecies rank. In addition, we highlight that the conceptual oversights result in incorrect and misleading information for decision makers that can have profound impact on the conservation of taxa. Our primary intention here is to focus on furthering our collective understanding of how genomic analyses and evolutionary relationships should be interpreted, and their limitations for governing changes in infraspecific taxonomy.

Puckett et al. (2021) accepted a lack of supported evidence for the genetic distinctness of *N. m. atristriatus* (e.g., a lack of strong nodal support of evolutionary relationships recovered from a phylogenetic tree) as conclusive evidence for synonymy of the three southern subspecies of least chipmunks (*N. m. atristriatus*, *N. m. operarius*, and *N. m. caryi*; hereafter Southern group). We agree with Puckett et al. (2021) that the evidence indicates that *N. m. atristriatus* is genetically aligned as a member of the Southern group. The relationship between *N. m. atristriatus* and other populations in the Southern Rocky Mountains is expected based on biogeography (Sullivan 1985). However, this relationship has no bearing on the subspecific status of *N. atristriatus*. It simply reflects that these individuals share a more recent common ancestor than other populations of *Neotamias*. Second, even if the recovered phylogenetic pattern was consistently well-supported paraphyly or polyphyly among subspecies within the Southern group (i.e., evolutionary non-independence that suggests either that interbreeding is still occurring or that not enough time has passed for populations to exhibit fixed genetic differences), it would still not be appropriate to invalidate subspecies status. Subspecies are well-established as potentially interbreeding units of analysis and represent taxa on the continuum of the formation of species (Wilson and Brown 1953; Padial et al. 2010; Patton and Conroy 2017). Lack of strong support for a relationship does not signal strong support for the alternative (unless the alternative is strongly supported). The authors did not provide hypotheses or predictions to be tested, but the implicit null hypothesis they tested was that *N. m. atristriatus* is not a valid subspecies. Thus, their interpretation that *N. m. atristriatus* is not distinct from the other members of the Southern group opens them to a classic type II statistical error, wherein they accepted the null hypothesis as true based on the absence of information that the subspecies are different (Patten 2010; Patten and Remsen 2017).

None of the analyses used to assess distinction of *N. m. atristriatus*, including the mitogenome haplotype network, clustering analyses, and nuclear phylogenomic tree, provide any statistical support for independence or for non-independence. The Splitstree method for mitogenome haplotype network construction does not provide any statistical support for groups (Puckett et al. 2021 – Fig. 2), and is therefore only representative of the genetic distance between individuals (Huson et al. 2008); specimens of *N. m. atristriatus* appear to be grouped more closely to each other than to any other individuals of the Southern group, although distance values are not provided. The principal components clustering analyses do not provide K-values for number of clusters or 95% ellipses around discrete groups (Puckett et al. 2021 – Fig. 4). The first two components of this ordination within *N. minimus* only account for 9.3% of the observed genetic variation, indicating considerable variation among these taxa was not reported. The nuclear concatenated phylogenomic tree provides no bootstrap support for any relationship within the Southern group clade or even for monophyly of the Southern group (Puckett et al. 2021 – Fig. 6). Lack of support values means we can draw no conclusions about the strength of relationships among individuals within this clade. Given this ambiguity, we cannot conclude that *N. m. atristriatus* is *not* distinct.

Puckett et al. (2021) viewed the unequivocal grouping of the three Southern subspecies as support for their primary conclusion that lack of reciprocal monophyly between *N. m. atristriatus* and other subspecies justifies synonymy with *N. m. operarius*. However, reciprocal monophyly is an inappropriate criterion for validating subspecies (Braby et al. 2012). Reciprocal monophyly occurs when two or more clades are each monophyletic (genetically unique) with respect to the other, and given the genetic data being analyzed. This condition forms the basis of both the genealogical and phylogenetic species concepts (Wheeler and Meier 2000; de Quieroz 2007). At the genome scale, reciprocal monophyly would be indicative of a lack of gene flow between biological species. By extension, reciprocal monophyly is explicitly not an acceptable criterion for defining subspecies (Patten 2015). Subspecies are characterized by heritable diagnostic traits including morphological or molecular differences that vary in frequency between geographically discrete but potentially interbreeding units of analysis (Hennig 1966; Patten 2010; Patton and Conroy 2017). Thus, gene flow is expected among subspecies, which would result in a lack of reciprocal monophyly (Patten 2010, Patten and Remsen 2017). Put simply, subspecies are not expected to be reciprocally monophyletic. However, interpretation becomes more complicated when considering that the extent of genetic differentiation between closely related taxa depends on multiple factors. The length of time that taxa have diverged from one another is ultimately reflected by how resolved genetic relationships are, but this also depends on both functional and stochastic processes that cause some parts of a genome to resolve as reciprocally monophyletic faster than others (Funk and Omland 2012). The choice of data is therefore consequential for the power to resolve relationships. Phylogeny estimation might recover well-supported reciprocal monophyly between two recognized subspecies from a given genetic locus. Conversely, even fully reproductively isolated species may exhibit a lack of reciprocal monophyly at a given locus due to processes that include incomplete lineage sorting and ancient hybridization, both common phenomena among mammals, and in particular among western chipmunks (Sullivan et al. 2014). As an example, Puckett et al. (2021) did not recover well supported reciprocal monophyly for *N. alpinus*, based on their exon data, although this is a recognized species based on more rapidly evolving loci coupled with other diagnostic characters (Rubidge et al. 2014). As such, although reciprocal monophyly is commonly used to indicate evolutionary independence of species under several species concepts, it is not a relevant criterion for assessing the validity of infraspecific taxonomy (Braby et al. 2012, Patten 2015).

A lack of gene flow between populations can also be achieved simply through strict geographic isolation, and isolation is a key criterion for diagnosing independent evolutionary trajectories (Franklin 1980; Sobel et al. 2010). From its most basic perspective, strict geographic isolation means that inheritance of genotypes from generation to generation, along with epigenetic factors (genotype-environment interactions) and any local adaptive pressures, is not influenced by any immigration and subsequent reproduction of related individuals from separate populations. Given isolation and local environmental conditions, the phenotype of a population will diverge through various evolutionary mechanisms including neutral genetic drift (particularly in small, declining, or demographically unstable populations) and the adaptive processes of natural selection in response to unique and particularly extreme environments. All of these dynamics are reflected by the ecology of *N. m. atristriatus* (Frey and Boykin 2007). Geography and geographic isolation are inextricably linked to the concept and delineation of subspecies (Vignieri et al. 2006, Patten 2010). As such, geographic isolation of *N. m. atristriatus* for an extended timeframe, with evidence from both the divergence time estimates of Puckett et al. (2021) and by the relatively well-resolved zoogeographic history of isolation and connectivity among the southwestern sky islands (e.g., Patterson 1982; Frey et al. 2007; Hope et al. 2016; not discussed by Puckett et al. 2021) constitute primary lines of evidence for uniqueness of this subspecies. *Neotamias m. atristriatus* diverged from other subspecies of the Southern group between 190 thousand years ago (kya; Puckett et al. 2019) and 824 kya (Puckett et al. 2021), two mean divergence estimates based on nuclear species-tree analysis and mitogenome phylogeny reconstruction (under a Yule tree prior), respectively. The predicted distribution of *N. minimus* during the Last Glacial Maximum (~18 kya) also demonstrates isolation of *N. m. atristriatus* from other Southern group subspecies (Puckett et al. 2021 – Fig. 8). All of the evidence presented supports prolonged isolation of *N. m. atristriatus* on an independent evolutionary trajectory. It then may be considered a matter of philosophical differences as to whether such a taxon represents a distinct subspecies (e.g., King et al. 2006; Ramey et al. 2007; Weckworth et al. 2015; Cronin et al. 2015), or indeed a distinct species (de Queiroz 2020). In our view, the question of taxonomic validity of *N. m. atristriatus* therefore becomes: Have extended isolation, unique population ecology, and an associated independent evolutionary trajectory resulted in diagnosability of this subspecies?

There exists an extended literature on the various definitions of subspecies (for thorough reviews see Haig et al. 2006; Remsen 2010; Patton and Conroy 2017; and citations therein). Puckett et al. (2021) used a definition of subspecies devised specifically for cetaceans by Taylor et al. (2017:174): "…a population, or collection of populations, that appears to be a separately evolving lineage with discontinuities resulting from geography, ecological specialization, or other forces that restrict gene flow to the point that the population or collection of populations is diagnosably distinct." With regards to diagnosability, Taylor et al. (2017) invoked the definition of Archer et al. (2017:104) as "…a measure of the ability to correctly determine the taxon of a specimen of unknown origin based on a set of distinguishing characteristics". Subsequently, Puckett et al (2021:11) stated (including emphasis) that "it is unclear what diagnosable, heritable character could be used to correctly determine that a least chipmunk specimen of *unknown origin* was *N. m. atristriatus*." We argue that origin matters, given that subspecies are geographic variants, and particularly among mammals, subspecies are described based on their geographic distribution coupled with diagnosable characters (Hall 1981; Remsen 2010). Archer et al. (2017) did not provide insight to why diagnosis of cetaceans must be based on a specimen of unknown origin, but we presume that it reflects whale migration, and this is supported by their citation of Brambilla et al. (2010) with reference to diagnosability of migratory bird subspecies. This definition is not relevant for terrestrial non-migratory small mammals. The geographic origin of *N. m. atristriatus*, coupled with morphological or molecular characters, would allow for recognition of this subspecies. For instance, even photographs taken with remote cameras allow for accurate diagnosis of *N. m. atristriatus* when paired with information about location (McKibben and Frey 2021). Origin aside, the statement of unclear diagnosability by Puckett et al. (2021) is misleading to decision-makers. This is coupled by a statement in the introduction that "…considerable scientific uncertainty surrounds the validity of *N. m. atristriatus* as a subspecies" (Puckett et al. 2021:2), which is dismissive of the statistical support for morphological and ecological distinction of this taxon, based on previously published data. We contend that *N. m. atristriatus* is diagnosable via multiple characters investigated since its description: 1) significant genetic differentiation measured by the fixation index (F_{ST} values) between *N. m. atristriatus* and other Southern group subspecies (Puckett et al. 2021:Supporting Information Appendix S1); 2) unique mitochondrial diversity (Puckett et al. 2021:11); 3) unique pelage, unique cranial and bacular morphology, and unique allozymes (genetic variants; Sullivan 1985); and 4) unique ecological habitat associations (Sullivan 1985). For context, we also point out that subspecies are based on population level diagnosability, not diagnosability of each individual in a population (Patten 2015). This taxon *is* diagnosable.

Given the lack of phylogenetic resolution recovered from exon capture data, Puckett et al. (2021) may have benefited by reporting additional analyses with their data, or minimally by discussing shortcomings, leaving the door open for further future analyses that might more accurately test hypotheses of uniqueness for *N. m. atristriatus* (Padial and Riva 2021). For instance, exon data are known to evolve more slowly than intron data and may not be most suitable for resolving the tips of the tree of life (Bi et al. 2012). Exon data are most beneficial for quantifying adaptive processes (Luikart et al. 2018), including divergence among taxa, through analysis of non-neutral outlier loci, but assessments of this variation were not presented. Finally, from an explicit conservation standpoint, methods have recently been developed for hierarchical assessment and designation of conservation units including not just evolutionary significant units but also management units based on neutral loci and adaptive units based on loci under selection (Funk et al. 2012; Barbosa et al. 2018; Hohenlohe et al. 2021). Although none of these units are considered for mammalian listing under the ESA, they would surely bolster the importance of a recognized subspecies such as *N. m. atristriatus* in the context of the entire species.

We conclude that, rather than invalidating *N. m. atristriatus*, the results of Puckett et al. (2021) actually augment prior research demonstrating the validity of *N. m. atristriatus* as a subspecies. It has experienced long-term geographic isolation, and it is morphologically, genetically, and ecologically distinctive. We therefore recommend that *N. m. atristriatus* be considered for listing under the ESA at the subspecies level. The misinterpretation of genomic data as we have described can matter for endangered species listing. In some cases taxonomic disputes have ostensibly even been used in attempt to thwart or cast doubt on ESA listings (Vignieri et al. 2006). The proposal to list *N. m. atristriatus* as a subspecies under the ESA is currently under a 60-day public review period (USFWS 2021), which makes the discussion about validity of its taxonomic status of critical importance. Since its inception, the ESA has always allowed listing of species and subspecies as these are formally recognized taxonomic entities (Haig et al. 2006). More recently, policy has also allowed the listing of Distinct Population Segments (DPSs) of vertebrates. DPSs are defined based on discreteness and importance relative to the remainder of the taxon, which means that interpretation of taxonomy can influence recognition of a DPS (Haig and D'Elia 2010). Thus, although Puckett et al. (2021) promoted their results as strong support for the Sacramento Mountains population forming a unique DPS, their overarching conclusion that *N. m. atristriatus* is an invalid taxon casts doubt on the current evidence presented as a basis for listing. Our account of the various misinterpretations of Puckett et al. (2021) reflects many of the same issues noted from other molecular genetic studies that have tested the validity of subspecies (e.g., Vignieri et al. 2006, Patten 2015). *Neotamias m. atristriatus* is a Linnean trinomial taxon that was described by a professional taxonomist (Bailey 1913) and has been validated by many subsequent analyses of its genetics, morphology, and ecology (Sullivan 1985; Sullivan and Peterson 1988). It therefore has higher priority for listing; DPSs are more prone to litigation and prolonged interpretation that could stall conservation efforts (Haig and D'Elia, 2010). Importantly, subspecies are a formal biological rank that may be contested on biological grounds, but cannot be rescinded through legal legislation, as can DPSs (Haig et al. 2006).

We present this case study in response to a more general rapid expansion of genomic methods for assessing imperiled taxa associated with ESA listing. Such studies are inherently "applied research" and reach multiple stakeholders with variable levels of expertise for interpreting these complex datasets. Importantly, for those stakeholders not accustomed to translating genomic jargon, such data and analyses are not easily associable with their relevance to the ecology, biogeographic history, and contemporary demographic trends of the taxon of interest. Thus, some may rely on the conclusions presented without the knowledge of theory and molecular methods necessary to rigorously decipher data and results. Greater integration among disciplines is imperative (Godfray and Knapp 2004; Padial et al. 2010). Molecular ecologists that have adopted genomic methods should invest in more comprehensive understanding of the biology of the study taxon and system. Studies focused on taxonomic assessments would benefit from collaboration with taxonomists; molecular ecology and taxonomy are not equivalent disciplines (Pruett and Winker 2010). And, extra care should be made to clearly explain what each analysis can or cannot confirm about the question of interest. Decisions by management agencies based on academic interpretations of complex datasets can be consequential for the maintenance of biodiversity. It is therefore equally important that decision makers have the information they need to accurately assess the findings of genomic analyses. We suggest that second opinions from unbiased experts of both the ecology and evolution of the focal taxon could save time and resources towards conservation legislation. Finally, journal editors should assure that data and comprehensive methods associated with taxonomic studies that relate to listing decisions be made available so that they can be evaluated for reproducibility (e.g., Fanelli 2018; Gilbert et al. 2021). In addition, journal editors considering these types of studies should not accept for publication interpretations of reciprocal monophyly for qualifying subspecies status. Although we vigorously disagree with their conclusions, Puckett et al. (2021) have provided the first focused genomic assessment of relationships among Southern subspecies of least chipmunks. Their study has quantified divergence of *N. m. atristriatus* based on two genomic datasets and limited sampling of this imperiled subspecies. Undoubtedly, future studies will benefit from their contributions for appropriate protections of declining wildlife.

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