

1 Genomic resolution of cryptic species diversity in chipmunks

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16 **ABSTRACT**

17 Discovery of cryptic species is essential to understanding the process of speciation and assessing
18 the impacts of anthropogenic stressors. Here, we used genomic data to test for cryptic species
19 diversity within an ecologically well-known radiation of North American rodents, western
20 chipmunks (*Tamias*). We assembled a *de novo* reference genome for a single species (*Tamias*
21 *minimus*) combined with new and published targeted sequence-capture data for 21,551
22 autosomal and 493 X-linked loci sampled from 121 individuals spanning 22 species. We
23 identified at least two cryptic lineages corresponding with an isolated subspecies of least
24 chipmunk (*T. minimus griseescens*) and with a restricted subspecies of the yellow-pine chipmunk
25 (*T. amoenus cratericus*) known only from around the extensive Craters of the Moon lava flow.
26 Additional population-level sequence data revealed that the so-called Crater chipmunk is a
27 distinct species that is abundant throughout the coniferous forests of southern Idaho. This cryptic
28 lineage does not appear to be most closely related to the ecologically and phenotypically similar
29 yellow-pine chipmunk but does show evidence for recurrent hybridization with this and other
30 species.

31

32 **KEYWORDS**

33 speciation; hybridization; introgression; phylogenomics; *Tamias*; *Neotamias*

34

35 INTRODUCTION

36 Species are fundamental units of biodiversity, yet the basic task of species discovery often
37 remains incomplete, even in well-studied taxonomic groups. Whereas some of these difficulties
38 stem from philosophical disagreements on species delimitation (de Queiroz 2007), a far more
39 important source of uncertainty involves the misidentification of cryptic lineages that are
40 phenotypically or ecologically similar to known species (Bickford et al. 2007; Struck et al.
41 2018). Identifying cryptic diversity is fundamental to understanding both the process of
42 speciation and the conservation of species. The emerging threats of accelerated climate change
43 and habitat loss have only increased the urgency of discovering and more fully accounting for
44 patterns of global biodiversity (Bickford et al. 2007; Delic et al. 2017).

45 Species delimitation has historically relied on morphological differences. However,
46 molecular data provide evidence that morphological divergence does not always coincide with
47 reproductive isolation. Moreover, the evolutionary history of speciation can be further obscured
48 by hybridization and introgression (Lamichhaney et al. 2017; Fišer et al. 2018). Over the past
49 decade, both theoretical and methodological approaches for genetically based species
50 delimitation have seen considerable progress (Yang and Rannala 2010; Fujita et al. 2012;
51 Edwards and Knowles 2014; Mirarab et al. 2014a; Yang 2015; Mirarab et al. 2016; Luo et al.
52 2018). In conjunction, advances in high-throughput DNA sequencing provide the ability to
53 reconstruct complex evolutionary histories using sophisticated statistical and phylogenetic
54 approaches that can detect and quantify the extent of gene flow during speciation (Green et al.
55 2010; Blischak et al. 2018).

56 Chipmunks of western North America provide a rich system to study speciation and species
57 delimitation across a rapid diversification characterized by ecological adaptation, phenotypic

58 convergence, and overlapping geographic distributions. Of the 25 or 26 (see Burgin et al. 2018)
59 currently recognized chipmunk species (Sciuridae: *Tamias*; but see Patterson and Norris 2016),
60 two are widely distributed lineages occupying central and eastern Asia (*T. sibiricus*) or eastern
61 North America (*T. striatus*; Hall 1981). The remaining 23 species (subgenus *Neotamias*)
62 comprise a recent radiation of habitat specialists and generalists occupying the diverse
63 ecosystems of western North America (Heller 1971; Patterson 1981; Sullivan et al. 2014).
64 Specialization among chipmunks has resulted in strong niche partitioning by habitat and
65 competitive exclusion among co-distributed species (Brown 1971; Heller 1971). Although
66 ecological associations broadly tend to track species boundaries, these associations can break
67 down in sympatry, with chipmunks that occupy similar or transitory habitats showing phenotypic
68 convergence (e.g., in size and pelage coloration) between non-sister species. As a consequence,
69 internal genital bones often provide the only morphological character that is strongly diagnostic
70 of species boundaries (White 1953). Divergence in genitalia is most pronounced in the male
71 genital bone, the baculum (*os penis*), which evolves rapidly among chipmunk species (Callahan
72 1977; Sutton and Patterson 2000; Sullivan et al. 2014) likely due to strong sexual selection
73 (Eberhard 1985; Simmons 2014). Bacular divergence was long thought to underlie strong
74 reproductive barriers between chipmunk species (Patterson and Thaler 1982). However, more
75 recent work has shown that some hybridization occurs between species (see Sullivan et al. 2014
76 for review). In most cases, levels of nuclear introgression have appeared to be relatively low
77 overall (but see Ji et al. 2021), with the persistence of genetically well-defined species
78 boundaries that largely correspond with established taxonomy based on ecological associations
79 and diagnostic genitalia (Hird and Sullivan 2009; Hird et al. 2010; Good et al. 2015; Bi et al.
80 2019; Sarver et al. 2021).

81 Genetic studies have also suggested several potential cases of cryptic diversity in this
82 radiation (Demboski and Sullivan 2003; Reid et al. 2012; Sullivan et al. 2014) and the phylogeny
83 of western chipmunks remains unresolved. One potential source for cryptic diversity lies within
84 the widespread least chipmunk (*T. minimus*). For example, *T. m. grisescens* (the Coulee
85 chipmunk, hereafter *grisescens*) is a small, light gray subspecies of *T. minimus* that occupies a
86 restricted range from the Channeled Scablands of central Washington that was identified by Reid
87 and colleagues (2012) as a potential cryptic lineage based on four nuclear loci and mitochondrial
88 DNA (mtDNA).

89 Another potential example of cryptic diversity occurs within the yellow-pine chipmunk (*T.*
90 *amoenus*), a widespread species associated with xeric forests throughout western North America.
91 Blossom (1937) described a subspecies of *Eutamias amoenus* (i.e., the Crater chipmunk, *T.*
92 *amoenus cratericus*, hereafter *cratericus*) as occurring on the periphery of a series of relatively
93 recent lava flows (~10 kya) from Craters of the Moon National Monument and surrounding area
94 of central Idaho. This darker pelage variant of the yellow-pine chipmunk was assumed to reflect
95 local adaptation on the black lava flows of southern Idaho, with rapid transition to more brightly
96 colored *T. amoenus* morphs found in adjacent xeric forest habitats of the region (Figure 1).
97 However, comparison of bacula by White (1953) and Sutton (1982, including the baubellum, the
98 female genital bone; *os clitoris*), of multiple *Tamias* species concluded that *cratericus* may be
99 more closely related to other chipmunk species (*T. umbrinus* or *T. ruficaudus*), based on similar
100 genital morphology and nearby geographic proximity. Finally, the mitochondrial genome
101 sequenced from *cratericus* appears to be fairly divergent from other species, and has been
102 detected at an additional locality to the north of Craters of the Moon (Demboski and Sullivan

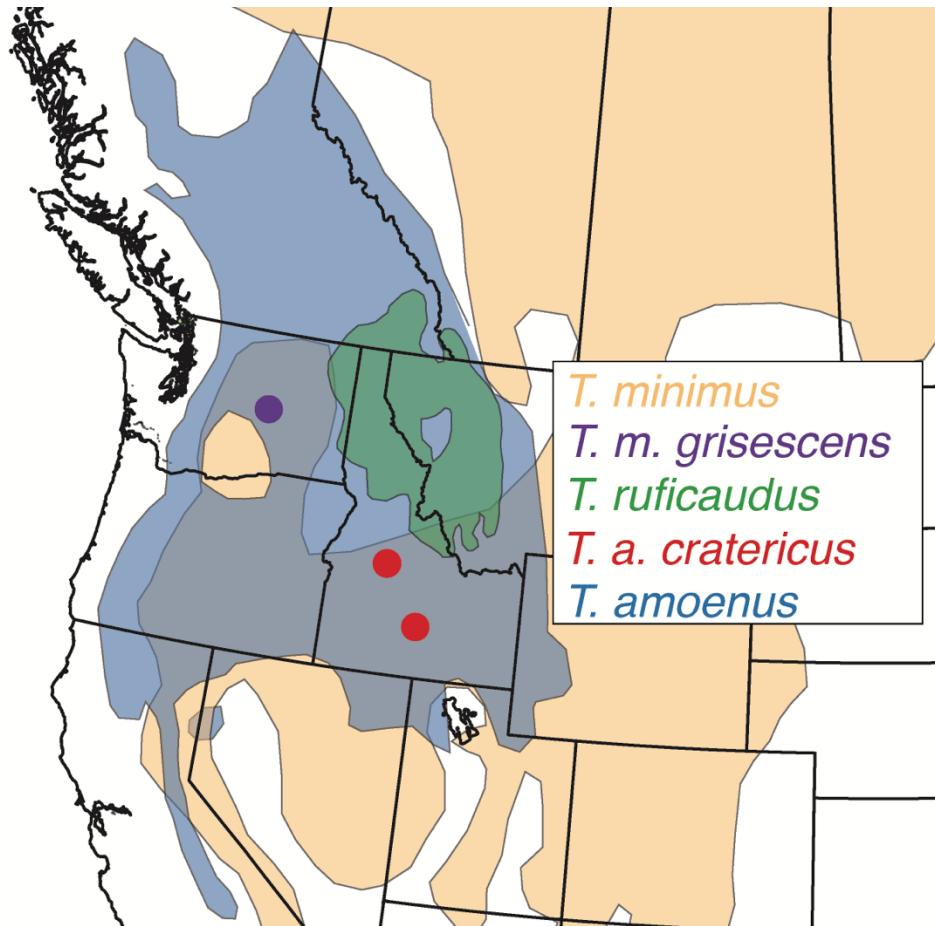


Figure 1 – Two potential cryptic species of chipmunk. *T. m. grisescens* (Purple) occupies a restricted range from the Channeled Scablands of central Washington. *T. a. cratericus* (Red) is described as a locally adapted form of the yellow-pine chipmunk (*T. amoenus*).

103 2003) suggesting that *cratericus* may be more widespread (Reid et al. 2012).

104 Here, we use linked-read sequencing to generate a reference genome for the least chipmunk
105 (*T. minimus*). We then use new and published targeted capture sequencing data from 21,551
106 autosomal and 493 X-linked loci, and complete mitochondrial genomes to infer the phylogenetic
107 relationships among 22 described chipmunk species, including the enigmatic *cratericus* and
108 *grisescens* chipmunks. We then use additional population-level sequencing to characterize the
109 geographic range and evolutionary history of *cratericus* relative to three other potentially co-
110 distributed chipmunk species.

111

112 MATERIALS AND METHODS

113 SAMPLING AND ETHICS STATEMENT

114 To assess genus-wide diversity, we combined extensive fieldwork with collections from several
115 natural history museums to obtain samples from 14 western chipmunk species and the eastern
116 chipmunk, *Tamias striatus*, as an outgroup (Supplementary Table S1). We combined data from
117 these samples with published data (Bi et al. 2019; Sarver et al. 2021) from seven additional
118 species of western chipmunks resulting in broad sampling covering 22 of the 26 described
119 species of chipmunk (57 individuals). Not included were samples of *T. solivagus*, *T. durangae*,
120 and *T. bulleri* (all western chipmunk species endemic to Mexico), and the Eurasian *T. sibiricus*.

121 To better characterize the geographic and ecological extent of *cratericus*, we collected an
122 additional 64 individuals from 10 populations throughout central Idaho covering a broad range of
123 habitats from the lava flows of Craters of the Moon up to the subalpine zone. All animals
124 sampled for this project were collected following procedures approved by the University of
125 Montana Animal Care and Use Committee (protocol numbers AUP 042-16JGDBS & 022-
126 10JGDBS). We used acceptable field collecting methods, conducted according to the
127 recommendations of the American Society of Mammalogists Animal Care and Use Committee
128 (Sikes and Mammalogists 2016).

129

130 GENOME SEQUENCING AND ASSEMBLY

131 Liver tissue from a female *T. minimus* was frozen in liquid nitrogen immediately after euthanasia
132 and sent to the DNA Technologies and Expression Analysis Core at the UC Davis Genome
133 Center for DNA extraction and library preparation. High molecular weight genomic DNA was
134 isolated following Jain et al. (2018). Briefly, 40 mg of flash frozen liver tissue was homogenized

135 by grinding in liquid nitrogen and lysed with 2 ml of lysis buffer containing 10 mM Tris-HCl pH
136 8.0, 25 mM EDTA, 0.5% (w/v) SDS and 100µg/ml Proteinase K. The lysate was cleaned with
137 equal volumes of phenol/chloroform using phase lock gels (Quantabio Cat # 2302830). The
138 DNA was precipitated by adding 0.4X volume of 5M ammonium acetate and 3X volume of ice-
139 cold ethanol, washed twice with 70% ethanol, and resuspended in buffer (10mM Tris, pH 8.0).
140 The integrity of the high-molecular-weight genomic DNA was verified on a Pippin Pulse gel
141 electrophoresis system (Sage Sciences, Beverly, MA). Following DNA isolation, a 10X
142 Genomics Chromium library was prepared following the manufacturer's protocol and sequenced
143 on the Illumina HiSeq X platform by Novogene Corporation. We then used Supernova V2.0.0
144 (Weisenfeld et al. 2017) to generate a *de novo* genome assembly from the 10X Genomics
145 Chromium library sequence data using default settings with --maxreads=1135000000 (the
146 number of reads estimated to yield 56× coverage for an estimated genome of ~2.3 Gigabases;
147 Supplementary Table S2).

148

149 **EXON CAPTURE ENRICHMENT AND SEQUENCING**

150 We generated new genome-wide resequencing data from 112 individuals and 15 chipmunk
151 species (including *T. striatus* as an outgroup; Supplemental Table S1) using a custom in-solution
152 targeted capture experiment designed by Bi et al. (2019) to enrich and sequence exons (including
153 flanking introns and intergenic regions) from 10,107 nuclear protein-coding genes [9.4
154 Megabases (Mb) total and the complete ~16.5 kilobase (kb) mitochondrial genome]. This capture
155 strategy was iteratively developed from a series of genomic experiments. First, RNA-seq was
156 used to sequence and assemble transcriptomes from multiple tissue types sampled from a single
157 chipmunk species (*T. alpinus*), which served as the reference for exome capture probe design (Bi

158 et al. 2012). These transcriptome data were used to develop an array-based capture targeting
159 ~8,000 nuclear genes as well as the complete mitochondrial genome, resulting in ~6.9 Mb of
160 assembled exonic regions (including flanking introns and intergenic regions; Bi et al. 2012; Bi et
161 al. 2013; Good et al. 2015). Finally, an additional ~2.4 Mb of genic regions were identified from
162 the original transcriptome through AmiGO and NCBI protein databases and were added to the
163 capture design. Published data from Sarver et al. (2021; *T. canipes*, *T. cinereicollis*, *T. dorsalis*,
164 *T. quadrivittatus*, *T. rufus*, and *T. umbrinus*) were based on the earlier array-based design and
165 thus represent an overlapping subset of the genomic regions targeted here.

166 To localize the targeted genomic regions within the *de novo* *T. minimus* genome, we first
167 used BLAT to identify the best hit in the reference. We then sorted and filtered the contigs to
168 include only the best match hits, discarding contigs with equally likely hits across the genome.
169 To identify X-linked contigs in the genome assembly, we calculated the normalized female
170 versus male ratio of sequencing coverage for targeted regions mapping to each contig, which
171 should be approximately 2:1 for genes on the X chromosome.

172 Total genomic DNA was isolated from heart or liver tissue using Qiagen DNEasy DNA
173 blood and tissue kits. Illumina sequencing libraries were prepared for each sample following the
174 protocol of Meyer and Kircher (2010). Sequencing libraries were then enriched in solution using
175 probes designed as described above and manufactured by NimbleGen (SeqCap EZ Developer
176 kits). Hybridization reactions were performed in four separate equimolar pools of indexed
177 libraries. To assess capture efficiency, we first sequenced a subset of pooled libraries on an
178 Illumina MiSeq sequencer (150 bp paired-end reads) at the University of Montana genomics core
179 (Missoula, MT). Final pooled libraries were sequenced on an Illumina HiSeq 2500 (150 bp
180 paired-end reads) at the University of Oregon (Eugene, OR).

181 **ILLUMINA DATA PROCESSING AND GENOTYPING**

182 Raw read data were cleaned using the *HTStream* pipeline (available from
183 <https://github.com/s4hts/HTStream>; last accessed July 21, 2018), to trim adapters and low-
184 quality bases, merge overlapping reads, and remove putative PCR duplicates. Cleaned reads
185 were mapped to the reference genome using the BWA-MEM alignment algorithm as
186 implemented in BWA v0.7.15 (Li and Durbin 2009; Li 2013) with default options and then
187 sorted with *samtools* v1.4 (Li et al. 2009). Mapped reads were assigned read groups and
188 duplicate reads were identified post-mapping using Picard v2.5.0
189 (<http://broadinstitute.github.io/picard/>). Initial capture efficiency statistics were calculated using
190 QC3 v1.33 (Guo et al. 2014). Regions with insertions or deletions (indels) were identified and
191 realigned, and single nucleotide variants (SNVs) were called using UnifiedGenotyper within the
192 Genome Analysis Toolkit (GATK) v3.6 (McKenna et al. 2010; DePristo et al. 2011; Van der
193 Auwera et al. 2013) with the EMIT_ALL_SITES argument set. This resulted in a final VCF with
194 a call at every position. We then soft filtered based on minimum mapping quality ($MQ < 20$), a
195 minimum quality ($QUAL < 20$), a minimum quality by depth ($QD < 2$), and a minimum
196 sequencing depth ($DP < 5$), resulting in a final filtered single sample VCF file used for all
197 downstream analyses.

198

199 **PHYLOGENETIC INFERENCE AND SPECIES DELIMITATION**

200 To obtain sequence alignments for phylogenetic analyses, we constructed consensus sequences
201 of the targeted regions using the filtered VCF for each individual by injecting all confidently
202 called sites back into the original reference using *FastaAlternateReferenceMaker* within the
203 GATK. IUPAC ambiguity codes were inserted at putative heterozygous positions using the -

204 IUPAC flag. Remaining ambiguous positions (MQ < 20, QUAL < 20, QD < 2, DP < 5) were hard
205 masked (i.e., replaced with an “N”) using GNU awk and bedtools v2.25 (Quinlan and Hall
206 2010).

207 We used a two-tiered approach to infer phylogenetic relationships within *Tamias*. First,
208 we used a concatenated alignment of the targeted regions and 100 base pairs (bp) of flanking
209 sequence to estimate a single bifurcating phylogeny using a maximum likelihood (ML) search as
210 implemented in IQ-Tree v.1.5.5 (Nguyen et al. 2015). For all phylogenetic analyses, we ran
211 separate analyses for autosomal and X-linked regions. We use ModelFinder Plus (-m MFP)
212 (Kalyaanamoorthy et al. 2017) and Bayesian information criterion (BIC) to select the best
213 nucleotide substitution model for the concatenated data. To assess nodal support, we performed
214 1000 ultrafast bootstrap replicates (UFBoot) with the “-bb” command (Minh et al. 2013; Hoang
215 et al. 2017). Concatenation of multiple loci ignores coalescent stochasticity and can result in
216 erroneously high bootstrap support for nodes that may show substantial incongruence when
217 considered on a per locus basis (Kumar et al. 2012). Therefore, we also calculated gene
218 concordance factors (gCF) and site concordance factors (sCF) as implemented in IQ-Tree v.
219 2.0.6 (Minh et al. 2020a; Minh et al. 2020b). Gene concordance factors are the proportion of
220 inferred single locus trees (gene trees) that support a particular branch in the reference tree (ML
221 concat tree), whereas site concordance factors are the proportion of sites within the concatenated
222 alignment that support a branch in the reference tree.

223 To obtain gene trees, we extracted alignments from the targeted regions and 100 bp of
224 flanking sequence by combining all targets found within stepped 50 kb intervals using bedtools
225 v2.2.5 and combined regions by scaffold using AMAS (Borowiec 2016). This approach was
226 chosen to capture the spatial distribution of individual targets while ensuring we include enough

227 informative sites to infer independent phylogenetic histories. Although interval size is somewhat
228 arbitrary here, we considered a range of interval sizes and found smaller intervals (≤ 10 kb)
229 resulted in many intervals without targets and few informative sites. Likewise, much larger
230 interval sizes (≥ 100 kb) were more likely to conflate independent phylogenetic histories. For
231 each interval, we filtered positions with missing data for $>85\%$ of individuals using AMAS and
232 excluded intervals containing less than 1 kb of capture data. We then used IQ-Tree v.1.5.5 to
233 estimate local maximum likelihood trees with 1000 ultra-fast bootstraps (using -m MFP, -bb
234 1000), with *T. striatus* as the outgroup. Gene trees, when necessary, were unrooted using the R
235 package *ape* (Paradis et al. 2004). We then used unrooted gene trees to estimate a consensus
236 species tree using ASTRAL-III v5.6.3 (Zhang et al. 2018). Assuming sets of independent and
237 accurately estimated gene trees, ASTRAL estimates an unrooted species tree by finding the
238 species tree that has the maximum number of shared induced quartet trees with the given set of
239 gene trees (Mirarab et al. 2014a; Mirarab et al. 2014b).

240 Next, we estimated coalescent-based species trees with SVDquartets (Chifman and
241 Kubatko 2014) using 100,000 random quartets and 100 bootstrap replicates as implemented in
242 PAUP* v4.0a166 (Swofford 2003). SVDquartets assesses support for quartets of taxa directly
243 from site-pattern frequencies of variable sites only. This approach differs from summary
244 methods, such as ASTRAL, because it does not independently estimate gene trees, avoiding the
245 issue of gene-tree estimation error (Chifman and Kubatko 2015; Chou et al. 2015). For
246 SVDquartets, we extracted single nucleotide variants (SNVs) distanced at least 10 kb along the
247 genome and excluded sites with missing information for $>85\%$ of the individuals. For both
248 SVDquartets and ASTRAL analyses, species trees were estimated with and without assigning
249 species identities and using sites or intervals for autosomal and X-linked loci separately as

250 previously described. When applicable, we included the eastern chipmunk, *T. striatus*, as the
251 outgroup.

252 Finally, we estimated a species tree using the Bayesian program BPP version 4.4.1
253 (Rannala and Yang 2017; Flouri et al. 2018). BPP uses a full likelihood-based implementation of
254 the multispecies coalescent and accommodates uncertainty due to gene tree heterogeneity (both
255 the topology and branch lengths) and incomplete lineage sorting. We first performed preliminary
256 runs using a diffuse prior (shape parameter $\alpha=3$) to assess the fit of the prior means with our data.
257 For our model parameters, we assigned inverse-gamma priors to the population size parameter θ
258 [\sim IG (3, 0.002) with mean 0.00], and to the divergence times τ [\sim IG (3, 0.01) with mean 0.005
259 for the age of the root]. We assessed convergence based on consistency across 5 replicate
260 MCMC runs, using a different starting tree and seed for each. For each run, we generated
261 200,000 samples with a sample frequency of two iterations after a burn-in of 16,000 iterations.
262 While BPP can potentially analyze 1000's of genetic loci, we found that runtimes were excessive
263 even with our relatively modest genome-wide capture dataset. Therefore, we explored several
264 pruning options and settled on selecting the largest contig with a minimum interval distance of 1
265 Mb. This resulted in a sequence alignment consisting of 88 loci (ranging in size from \sim 1,000-
266 4,600 bp; median 2,343 bp) for a total alignment length of 209,725 bp.

267

268 **INFERENCE OF POPULATION STRUCTURE**

269 We plotted PC1 and PC2 from a principal components analysis (PCA) using the R package
270 SNPRelate (Zheng et al. 2012). To estimate individual co-ancestry, we performed ML estimation
271 of individual admixture proportions using the program ADMIXTURE v.1.30 (Alexander et al.
272 2009). We tested values of K (the number of population clusters) ranging from 1-6 under the

273 default settings in ADMIXTURE and selected the model with the lowest cross-validation error.
274 For both PCA and population cluster analyses, we used a filtered SNP dataset where we applied
275 LD-based pruning to filter our SNP dataset using PLINK v1.9 (Chang et al. 2015) to minimize
276 non-independence due to linkage. We used a 1 kb sliding window with a step size of 100 bp and
277 pairwise r^2 of 0.8 as a cut-off for removing highly linked SNPs. This resulted in a final set of
278 93,247 SNPs.

279

280 **INTROGRESSION AND PHYLOGENETIC NETWORKS**

281 An advantage of SVDquartets is that it detects deviations in site-pattern frequencies expected
282 under the multi-species coalescent by evaluating support for all three resolutions for each quartet,
283 similar to the ABBA-BABA test (Green et al. 2010; Durand et al. 2011). Therefore, we used the
284 software package HyDe (Blischak et al. 2018) to test for hybridization using the invariants
285 framework of SVDquartets. Here, the quartet with the majority of the support should correspond
286 to the species tree. With incomplete lineage sorting (ILS) and in the absence of introgression, the
287 two minor quartet topologies should show similarly low support. Alternatively, introgression will
288 lead to an imbalance of support towards the topology with the two taxa exchanging alleles as
289 sister taxa (Pease and Hahn 2015; Blischak et al. 2018; Kubatko and Chifman 2019). HyDe also
290 estimates γ , which is the parental contribution in a putatively hybrid genome, where a value of
291 0.5 would indicate a 50:50 genomic contribution from each parent.

292 We also modeled hybridization and ILS under the coalescent network framework as
293 implemented in PhyloNet 3.6.6 (Yu and Nakhleh 2015). Networks were computed under
294 Maximum Pseudo-Likelihood (*InferNetwork_MPL*) (Yu and Nakhleh 2015) using rooted
295 autosomal gene trees. We modeled 0 to 5 migration events, associated individuals to species

296 (option -a), and optimized branch lengths and inheritance probabilities to compute likelihoods
297 for each proposed network (option -o). We calculated Akaike information criteria corrected for
298 small sample sizes (AICc) and Bayesian Information Criteria (BIC) using the highest likelihoods
299 per run to compare the resulting networks (Yu et al. 2012; Yu et al. 2014). Networks were
300 visualized with IcyTree (<https://icytree.org>; last accessed Dec 2020).

301

302 **RESULTS**

303 **DRAFT GENOME ASSEMBLY AND SEQUENCE CAPTURE**

304 We generated ~217 Gb of *T. minimus* genomic sequence data using 10X Genomics technology
305 (Pleasanton, CA, USA) and assembled a 2.48 Gb draft genome with a contig N50 of 196.09 kb
306 and as scaffold N50 of 58.28 Mb, respectively (Supplementary Table S2). We then analyzed up
307 to 9.4 Mb of sequence capture data from 121 individuals, combining newly generated (112
308 individuals from 15 species) and published data (nine individuals from seven species; Bi et al.
309 2019; Sarver et al. 2021; Supplementary Table S1) for 21 western chipmunk species and the
310 outgroup *T. striatus*. Sequencing efforts produced ~4 million reads per sample with an average of
311 0.5% of targets showing no coverage. Approximately 75% of raw reads were unique, resulting in
312 an average target coverage of 33× across samples (range: 5–91×) with ~73% of targeted bases
313 sequenced to at least 10× coverage (Supplementary Table S3). Comparison of male versus
314 female coverage resulted in the identification of three contigs (79.7 Mb, 35.6 Mb, 5.9 Mb) in the
315 genome assembly that are likely on the X chromosome.

316

317 **THE *TAMIAS* PHYLOGENY**

318 We first estimated a genus-wide ML phylogeny from a concatenated set of 21,551 autosomal
319 (5,365,556 bp) and 493 X-linked (105,671 bp) loci from the combined capture datasets, as well
320 as an ML phylogeny based on the full mtDNA genome (Figure 2A; Supplemental Figure S1-S2).
321 The concatenated autosomal ML analysis recovered a fully resolved phylogeny with high
322 support for all branches (UFBoot > 90%) and is concordant with previous studies (Reid et al.
323 2012; Sullivan et al. 2014; Sarver et al. 2021). *Tamias m. grisescens* appeared as a distinct
324 lineage in these analyses and was not most closely related to other *T. minimus* samples.
325 Likewise, *cratericus* was a distinct, monophyletic lineage that was most closely related to *T.*
326 *ruficaudus* and not other *T. amoenus*. Within *cratericus*, we also recovered a deep split between
327 the two sampled localities (hereafter *cratericus* lineages A and B; Figure 2A). ML reconstruction
328 of the mitochondrial genome resulted in a different topology from the nuclear dataset
329 (Supplemental Figure S2) and was largely concordant with previous studies based on the
330 cytochrome *b* gene (Demboski and Sullivan 2003; Good et al. 2003; Good et al. 2008; Reid et al.
331 2012; Good et al. 2015; Sarver et al. 2017) with *cratericus* most closely related to *T.*
332 *quadrimaculatus*, and then to *T. minimus*. Our mitochondrial genome reconstruction also
333 recovered an individual from the putative *cratericus* lineage B (CRCM06-160; Supplemental
334 Figure S2) as having a *T. amoenus* mtDNA genome, consistent with introgression. The
335 multispecies-coalescent species trees estimated from ASTRAL (158 genes trees estimated from
336 50 kb genomic intervals) and SVDquartets (13,482 unlinked SNPs) was largely concordant with
337 the results of the concatenated ML analyses. Strong support was recovered for the branching
338 relationships among species groups within *Tamias* (ASTRAL posterior probabilities > 0.9 and
339 SVDquartets bootstrap support > 90). All analyses recovered both *grisescens* and *cratericus* as
340 distinct, monophyletic lineages. However, one notable difference across analyses was the

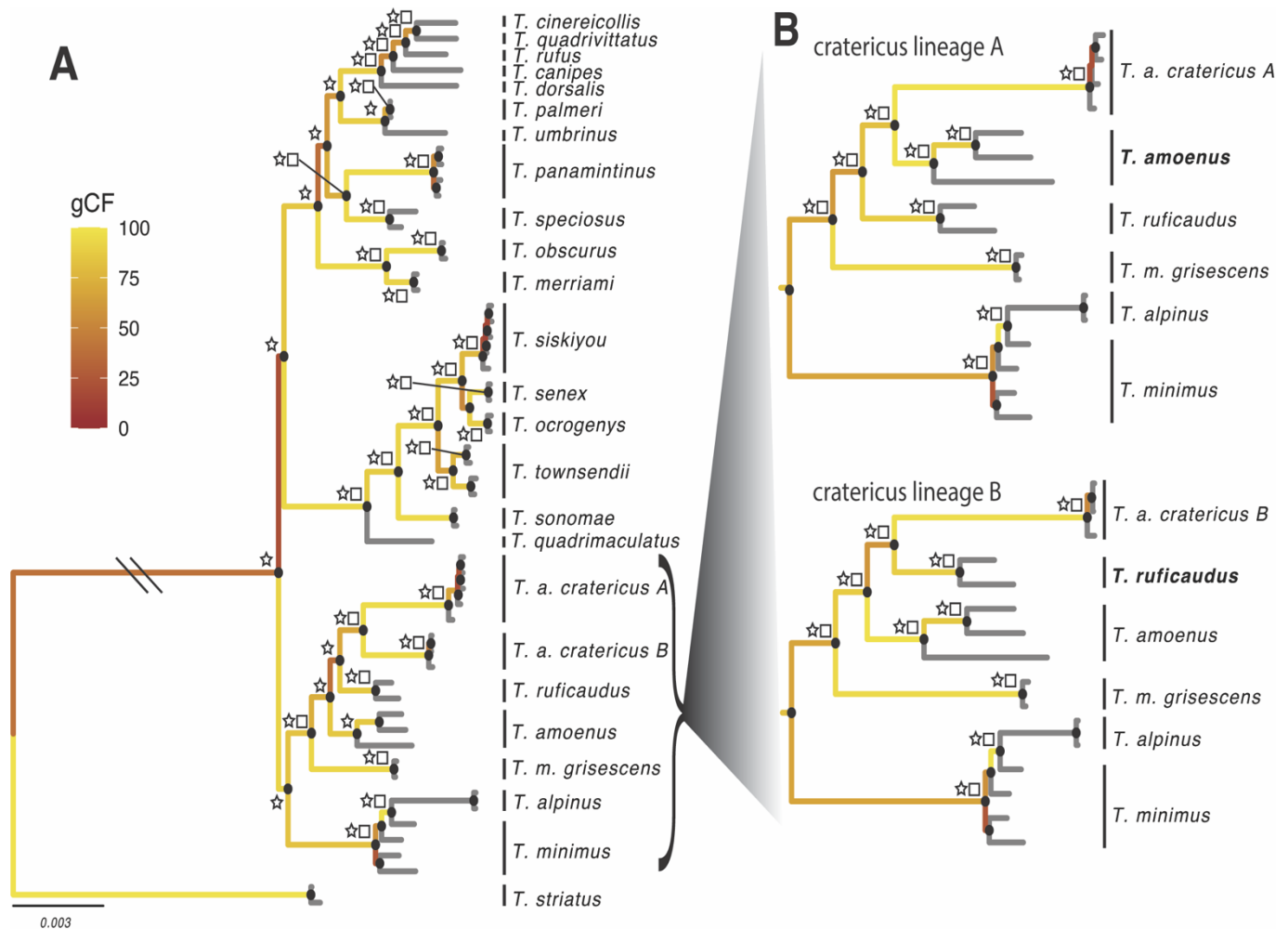


Figure 2 – Genome-wide phylogeny of *Tamias*. **A)** Concatenated ML tree estimated with IQ-Tree; Node labels represent: ASTRAL posterior probability equal to one (star), bootstrap greater than 90 (square), and bootstrap proportions greater than 90 (grey circles). Branches are colored according to gene concordance factor (gCF). **B)** ML reconstruction of the *amoenus-ruficaudus-cratericus* clade with the exclusion of *cratericus* lineage A and *cratericus* lineage B respectively.

341 relationship of *cratericus* with respect to *T. ruficaudus* and other *T. amoenus*. Both concatenated
 342 ML analyses and ASTRAL recovered *cratericus* as the sister lineage to *T. ruficaudus*, whereas
 343 SVDquartets recovered *cratericus* as more closely related to other *T. amoenus* but with weak
 344 support (Supplemental Figures S3-S6). Overall, the splits between *T. amoenus-cratericus-T.*
 345 *ruficaudus* were not well-supported and remained unresolved (Figure 2A; Supplemental Figure
 346 S1-S6).

347 Given the deep split within the *cratericus* clade and low support for the branching
348 structure, we hypothesized that there might be unequal ancestry between the two *cratericus*
349 lineages, *T. amoenus*, and *T. ruficaudus*. To explore this we re-analyzed these data while
350 excluding either *cratericus* lineage A or lineage B. When *cratericus* lineage B was excluded, we
351 consistently recovered *cratericus* as the sister lineage to *T. amoenus* with strong support. In
352 contrast, analyses excluding *cratericus* lineage A recovered *cratericus* as sister to *T. ruficaudus*
353 with strong support (Figure 2B).

354

355 **POPULATION GENOMICS OF *CRATERICUS***

356 To elucidate the evolutionary history and geographic extent of the cryptic *cratericus* lineages, we
357 sequenced an additional 64 chipmunks from an additional 12 localities (seven *cratericus*/*T.*
358 *amoenus* localities; five *T. minimus* localities; Figure 3) ranging from relatively low desert sage
359 scrub to higher elevation temperate coniferous forests throughout south central Idaho. Initial
360 qualitative assessment of gross bacular morphologies from these samples suggested that
361 *cratericus* may be more widespread in central Idaho, ranging north from the temperate
362 coniferous forest/sage scrub transitional zone of the Snake River Plain to the temperate/subalpine
363 zones of the Salmon River. Following the procedures above, we generated an alignment of
364 7,813,766 bp for autosomal loci and 175,884 bp for X-linked loci with >80% of individuals
365 genotyped at a minimum coverage of 5×.

366 The ML tree from the concatenated set of autosomal and X-linked loci were largely
367 congruent with the genus-wide analysis. *T. minimus* (minus *griseescens*) was monophyletic, with
368 *T. m. griseescens* as a distinct lineage that was more closely related to the *T. amoenus-cratericus-*
369 *T. ruficaudus* complex than to other *T. minimus*. Our expanded sampling substantially increased

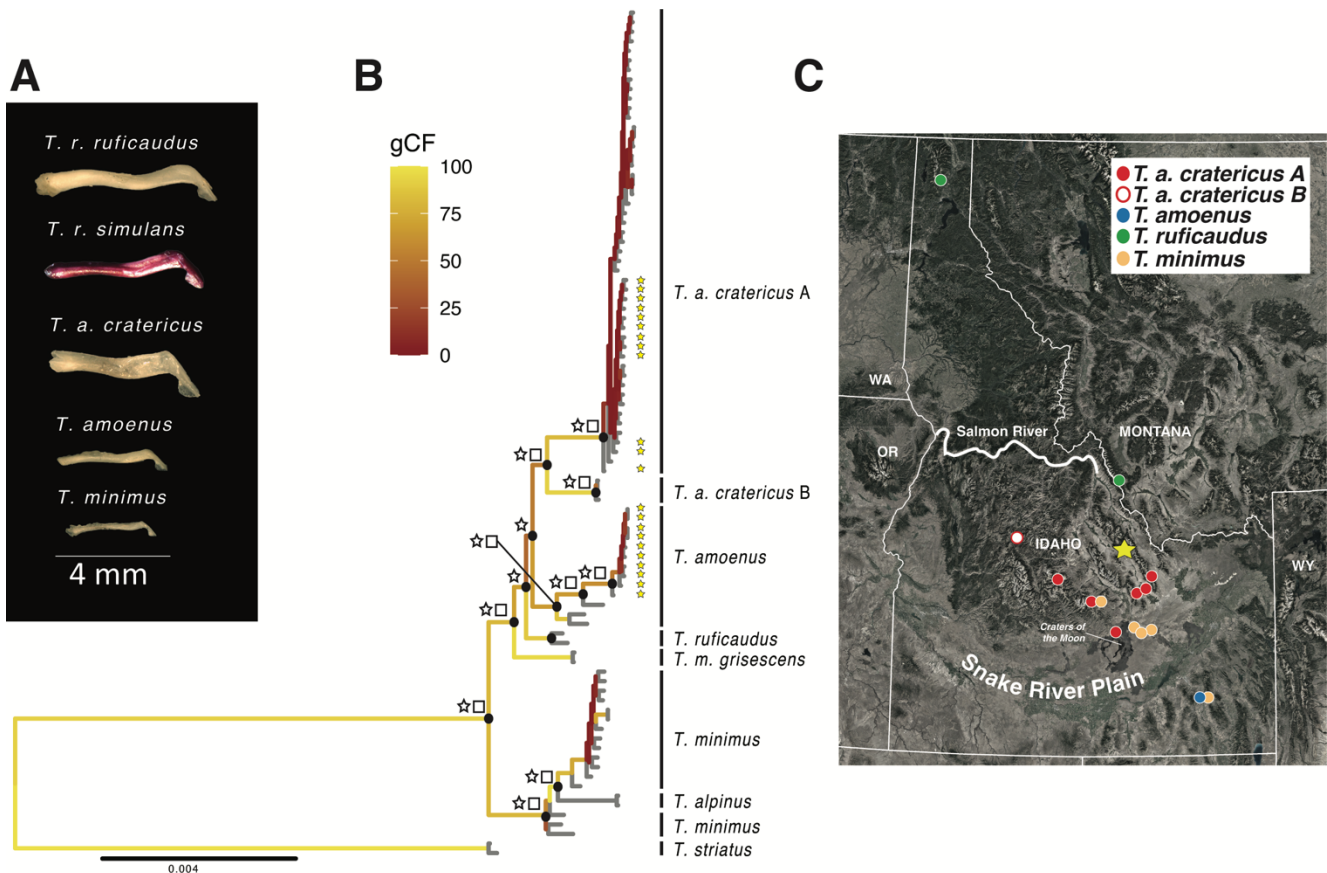


Figure 3 – ML concatenated phylogeny for *cratericus-amoenus-ruficaudus*. **A)** Representative bacular morphologies for the five taxa. **B)** Concatenated ML tree estimated with IQ-Tree for the full population level sampling of *cratericus*; Node labels represent: ASTRAL posterior probability equal to one (star), SVDquartets bootstrap greater than 90 (square), and bootstrap proportions greater than 90 (black circles). Branches are colored according to gene concordance factor (gCF). Yellow stars at the tips indicate *cratericus* and *T. amoenus* samples from the sympatric Iron Creek locality (yellow star on map). **C)** Sampling localities for population sampling. Note that our sampling also includes non-Idaho populations for *T. minimus*, *T. amoenus*, and *grisescens* that are not shown.

370 the diversity detected within *cratericus* lineage A, which was found at 6 additional localities to
 371 the near exclusion of *T. amoenus*. We again recovered a deep split within *cratericus*, with
 372 lineage B still represented by only a single locality (Figure 3; Supplemental Figure S7).

373 However, the most notable difference between this expanded subset and our genus-wide
 374 analysis was that *cratericus* now appeared sister to other *T. amoenus* (Figure 3B; Supplemental
 375 Figures S10-13). One potential source of phylogenetic discordance between the datasets may be
 376 driven by samples from one locality, known as Iron Creek (IC; yellow star; Figure 3C). At this

377 locality, both *cratericus* and standard *T. amoenus* bacular types were found to co-occur without
378 other obvious phenotypic or habitat differences. This was the only site where both forms were
379 found to be sympatric. Collectively, these results suggest that *cratericus* is distributed across the
380 coniferous forest of south central Idaho, north of the Snake River Plain to the near exclusion of
381 *T. amoenus*, save for one sympatric locality. At this site, we observed the maintenance of
382 genetically defined species boundaries despite some evidence for admixture between *cratericus*
383 and *T. amoenus* (see below).

384 Next, we evaluated if the change in the relationship between *T. ruficaudus*-*cratericus*-*T.*
385 *amoenus* was influenced by possible gene flow at the sympatric IC locality. Consistent with this,
386 *cratericus* was again sister to *T. ruficaudus* in a concatenated analysis when excluding all IC
387 samples. However, the species trees estimated from ASTRAL (158 genes trees estimated from
388 50 kb genomic intervals) and SVDquartets (17,594 unlinked SNPs) for the expanded subset
389 resulted in *cratericus* being sister to *T. amoenus*, regardless of whether IC individuals were
390 included (Supplemental Figures S10-13). Branch support increased when IC samples were
391 excluded but we still found a large degree of incongruence based on gCF and sCF. Finally, the
392 results of our BPP analyses were largely consistent across the 5 replicate runs using different
393 starting species trees, with 4 of the 5 runs converging on the same maximum *a posteriori*
394 probability (MAP) tree, with a posterior probability of ~100%. BPP recovered *cratericus* as
395 being sister to *T. ruficaudus* (Supplemental Figure S9).

396 ML reconstruction of mitochondrial genomes from the expanded subset also showed
397 evidence for mtDNA introgression between *cratericus* and *T. amoenus* (Supplemental Figure
398 S8). As with the genus-wide analysis, *cratericus* and *T. amoenus* were paraphyletic, but the
399 paraphyly in the mtDNA tree was not solely driven by discordance within the IC locality. One

400 *cratericus* mtDNA clade was composed of individuals from the majority of *cratericus* lineages A
401 and B but excluding IC, and was sister to a monophyletic *T. minimus* clade. The second major
402 group was composed of *T. amoenus*, including IC *T. amoenus* and *cratericus* from IC as well as
403 a single individual from lineage B (CRCM06-160 as in the genus-wide analysis; Supplemental
404 Figure S8).

405 Principal components of genetic variance (PCA) further supported a history of
406 hybridization and mixed ancestry between *T. amoenus*-*cratericus*-*T. ruficaudus*. PC1 largely
407 partitioned the most divergent lineage, *T. minimus*, from a cluster consisting of *cratericus*-*T.*
408 *amoenus*-*T. ruficaudus*. PC2 split *T. amoenus* and *cratericus*, with *T. ruficaudus* falling
409 intermediate. Cross-validation with ADMIXTURE suggested five population clusters that were
410 largely concordant with the PCA (Figure 4B; Supplemental Figures S14-15), although we note
411 that this analysis is not well-suited to partition genetic clusters at this scale of interspecific
412 divergence (Lawson et al. 2018). Interestingly, both methods showed a tendency to cluster
413 *cratericus* lineage B with *T. ruficaudus* and suggested a closer relationship between *cratericus*
414 and *T. ruficaudus* than to *T. amoenus*. However, we also detected a moderate proportion of
415 shared ancestry between sympatric (IC) *cratericus* and *T. amoenus*, likely driven by gene flow in
416 sympatry.

417

418 **PATTERNS OF INTROGRESSION AND NETWORK ANALYSES**

419 We tested for further evidence of introgression using multiple analyses based on the multispecies
420 network coalescent (PhyloNet), quartet-based analyses under the invariants framework (HyDe)
421 and estimates of admixture proportions based on the D-statistic (Green et al. 2010; Durand et al.
422 2011; Yu and Nakhleh 2015; Blischak et al. 2018). Collectively, these analyses supported

423 varying levels of recurrent gene flow between both *cratericus*-*T. ruficaudus*, and *cratericus* -*T.*
424 *amoenus*. PhyloNet supported a model of more ancient introgression, which has likely affected
425 the inferred split between *T. amoenus*-*cratericus*-*T. ruficaudus*. The best supported network
426 analysis (5 reticulations) suggested that *T. ruficaudus* and *cratericus* were sister lineages, with
427 reticulation between ancestral populations of *T. amoenus* and the *cratericus* lineage. Other less
428 well-supported reconstructions placed *T. amoenus* as more closely related to *cratericus* with a
429 complex pattern of recurrent gene flow between *cratericus* lineage A and B with both *T.*
430 *ruficaudus* and *T. amoenus* (Supplemental Figure S16). Quartet-based analyses of introgression
431 resulted in three significant triplet comparisons supporting hybridization (Supplemental Table
432 S4). First, *cratericus* lineage A showed shared ancestry between *T. amoenus* and *cratericus*
433 lineage B, consistent with a close relationship between *cratericus* lineages A and B and recent
434 hybridization between both lineages and *T. amoenus*. We also detected a pattern of shared
435 ancestry between both *cratericus* lineages (A and B) with *T. ruficaudus* and *T. amoenus*.
436 However, the proportion of shared ancestry was asymmetrical with respect to the amount of
437 introgression inferred from *T. amoenus* into either *cratericus* lineage (*T. amoenus*-*cratericus*
438 lineage A $\gamma = 0.344$; *T. amoenus*-*cratericus* lineage B $\gamma = 0.541$). Under the ABBA-BABA
439 framework, we evaluated phylogenetic patterns of shared SNVs between both *T. amoenus*-
440 *cratericus* and *T. ruficaudus*-*cratericus*. We found that most sites supported a pattern of
441 differential introgression between *cratericus* lineage A and both *T. amoenus* and to a lesser

442 extent *T. ruficaudus* relative to *cratericus* lineage B (Supplemental Figure S17; Supplemental
443 Table S4-S5).

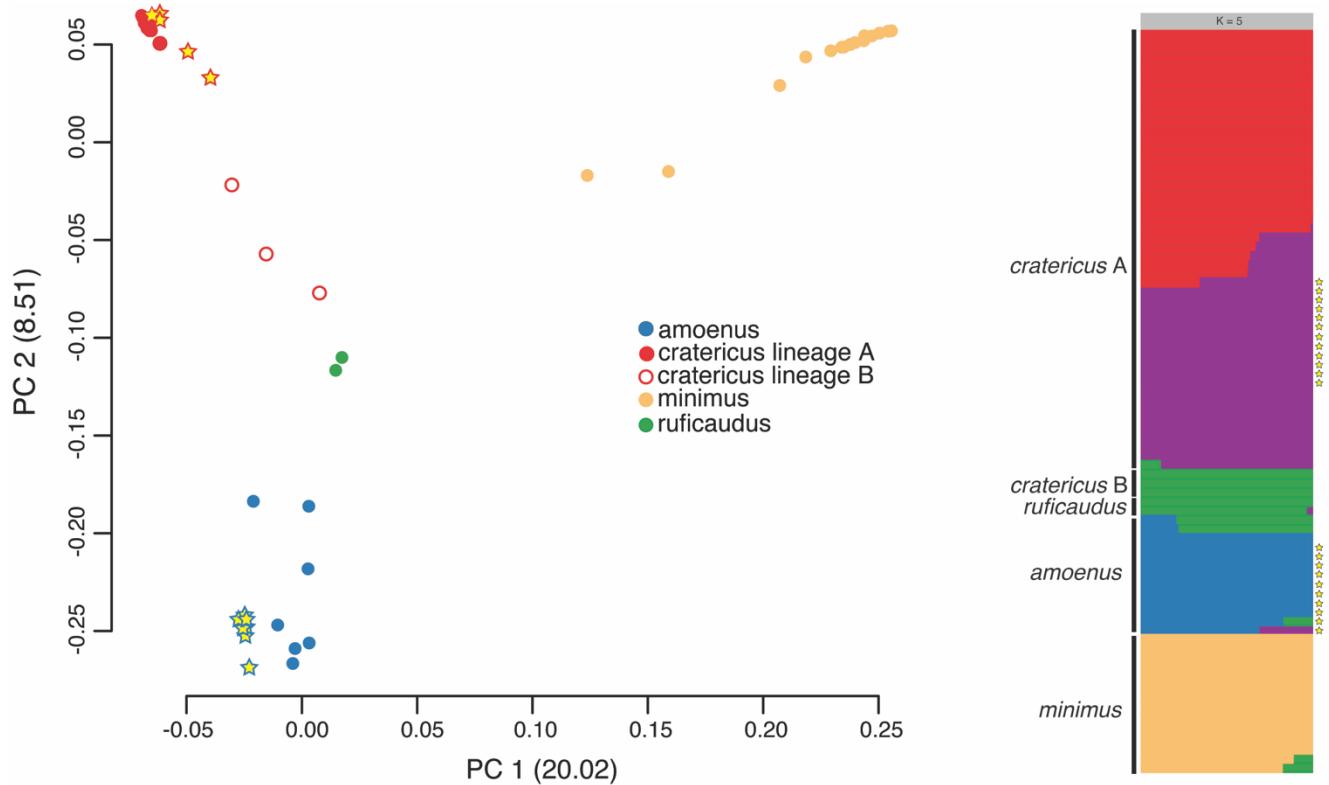


Figure 4 –Population structure between *cratericus*-*amoenus*-*ruficaudus* sampled. A) PCA results from SNPRelate. **B)** Admixture output for highest marginal-likelihood run at k=5. Stars indicate samples from the sympatric locality Iron Creek.

444 DISCUSSION

445 Discovery of cryptic species is fundamental to understanding the process of speciation and
446 cataloging global diversity, which is facing unprecedented pressures due to the emerging threats
447 of climate change and habitat loss. We generated a *de novo* assembled reference genome for a
448 western chipmunk species, *Tamias minimus*, and used new and published genome-wide targeted
449 capture sequencing data and whole mitochondrial genomes to test for cryptic speciation and
450 hybridization in western chipmunks. Our genus-wide analysis represents the most
451 comprehensive phylogenetic assessment of the western chipmunks to date and, we suggest,
452 reveals at least two new undescribed chipmunk species. Below we discuss the evolutionary

453 implications of our findings, focusing on western chipmunk diversity and the complex speciation
454 dynamics of the Crater chipmunk and other co-distributed species.

455

456 **SYSTEMATICS OF THE WESTERN CHIPMUNK RADIATION**

457 Due to their abundance and conspicuous nature, chipmunks have been the focus of a broad range
458 of biological studies including physiology (e.g., Levesque and Tattersall 2009), niche
459 partitioning and competitive exclusion (e.g., Grinnell and Storer 1924; Heller 1971), behavior
460 (e.g. Broadbrooks 1970), and responses to climate change (e.g., Moritz et al. 2008; Bi et al.
461 2019). Early phylogenetic studies using morphology and allozymes (Levenson et al. 1985),
462 host/ectoparasite (Jameson 1999), chromosomal (Nadler and Block 1962) and molecular data
463 (Piaggio and Spicer 2000; Piaggio and Spicer 2001) recognized three distinct clades within
464 *Tamias*: *T. sibiricus*, *T. striatus*, and the western North America species (arbitrarily classified as
465 either subgenera or genera; (e.g., Jameson 1999; Patterson and Norris 2016). Relationships
466 among the 23 currently recognized species within the western chipmunk clade (subgenus
467 *Neotamias*) have remained somewhat obscure due to a lack of resolution among internal nodes
468 and apparently rampant mitochondrial introgression (Piaggio and Spicer 2000; Reid et al. 2012;
469 Sullivan et al. 2014). Our analysis of western chipmunks represents the most comprehensive
470 genome-wide assessment of the group to date and included 22 of the 25 recognized species of
471 *Tamias*.

472 We recovered strongly supported, monophyletic species groups within the western
473 chipmunks that were in general agreement with some other studies (e.g., Reid et al. 2012).
474 However, one notable difference was the relationship between *T. ruficaudus*-*T. amoenus*-*T.*
475 *minimus*. In contrast to Reid et al. (2012), we recovered *T. ruficaudus* as being sister to either

476 *cratericus*, or to a clade composed of *T. amoenus-cratericus* (see discussion below) whereas
477 Reid et al. (2012) found *T. ruficaudus* sister to the enigmatic, and similarly distinct lineage
478 *griseus*. In addition, Reid et al. (2012) also recovered *T. amoenus* as sister to *T. minimus*,
479 whereas we found *T. minimus* as the sister lineage to the main *T. amoenus-T. ruficaudus-*
480 *cratericus-griseus* clade. Given a lack of resolution among internal nodes in previous studies,
481 this difference likely reflects the power afforded by much more extensive genetic sampling.

482 A major conclusion of our genomic study was the resolution of two cryptic lineages of
483 chipmunk. While some of the relationships among described species have been unclear until
484 recently, the identity of species as the fundamental units of diversity have remained fairly stable
485 over the last century. The vast majority of chipmunk species were described to some degree by
486 the late 19th and early 20th Centuries by the early naturalists Allen, Merriam, and others based
487 on phenotypic and ecological characteristics (for a review see Thorington Jr. et al. 2012).
488 Subsequent discussions have focused on determining if subspecific variation within some species
489 warrant species-level recognition (Patterson 1984; Patterson and Heaney 1987; Good et al. 2003)
490 or if some current species are actually geographically isolated populations of more widespread
491 forms (Piaggio and Spicer 2000; Piaggio and Spicer 2001; Rubidge et al. 2014). The putative
492 *griseus* lineage reflects these dynamics. Howell (1925) described the distinctive *T. m.*
493 *griseus* based on its pelage as a least chipmunk subspecies restricted to the Channeled
494 Scablands of central Washington. Reid et al. (2012) then identified this taxon as a potentially
495 cryptic lineage and found it consistently nested with *T. amoenus* based on limited nuclear and
496 mtDNA data. Although a formal description awaits additional geographic sampling, our data
497 clearly show that *griseus* does not fall within the considerable genetic diversity of the least
498 chipmunk (*T. minimus*) or other sampled species, and we propose that *griseus* is in fact a

499 novel, cryptic chipmunk species (i.e., *Tamias griseescens* following Howell 1925). Of note,
500 *griseescens* is much more genetically distinct from other chipmunk species than the Alpine
501 chipmunk (*T. alpinus*); a lineage that is paraphyletic with respect to *T. minimus* but has long been
502 recognized as a distinct species based on phenotypic divergence, ecological differentiation, and
503 reproductive isolation (Grinnell & Storer; Heller; Rubidge 2014; Bi et al. 2019). We also found
504 *griseescens* nested within *T. amoenus* in our mtDNA analyses, suggesting a history of mtDNA
505 introgression or incomplete lineage sorting (Supplemental Figure S3). While our study lacks the
506 sampling needed to fully investigate the evolutionary history of *griseescens* (two individuals from
507 the same locality), its range in central Washington could contribute to vulnerability due to habitat
508 loss, wildfire, and drought due to rapid climate change.

509 We also show that *cratericus* is likely a distinct chipmunk species that appears to be most
510 closely related to *T. ruficaudus*. Blossom (1937) first described *cratericus* as a duller, dark
511 variant of the yellow-pine chipmunk that is associated with the recent volcanic lava flows of
512 Craters of the Moon. The color morphology of *cratericus* rapidly transitions to a more brightly
513 colored pelage morph more like standard *T. amoenus* phenotypes and, we show, is found
514 throughout the adjacent xeric forest habitats of the region. This localized pelage presumably
515 reflects recent adaptation for crypsis on the black lava flows, a pattern that is also observed in
516 local populations of the Great Basin pocket mouse (*Perognathus parvus*) and pika (*Ochotona*
517 *princeps*) from Craters of the Moon (Blossom, 1937). More surprisingly, *cratericus* appears to be
518 the locally dominant form throughout central Idaho (see below).

519 Overall, we also found that most western chipmunk groups displayed a high degree of
520 discordance between the mtDNA phylogeny and the nuclear genome, consistent with previous
521 studies documenting recurrent mtDNA introgression layered across the history of this group

522 (e.g., Demboski & Sullivan, 2003; Sullivan et al. 2014; Sarver et al. 2017). Interestingly,
523 previous works have suggested distinct species boundaries with comparably low levels of
524 nuclear introgression relative to more rampant mtDNA introgression (Good et al. 2015; Sarver et
525 al. 2021; however, see Ji et al. 2021). From this perspective, the complex evolutionary history
526 and extensive nuclear gene flow observed between *cratericus* and both *T. amoenus* and *T.*
527 *ruficaudus* is noteworthy relative to other chipmunk studies to date.

528

529 **THE COMPLEX EVOLUTIONARY HISTORY OF *TAMIAS CRATERICUS***

530 Our data and analyses provide compelling evidence to support *cratericus* as at least one distinct
531 species. However, the full evolutionary history of this taxon appears to be obscured by a history
532 of rapid speciation and likely recurrent instances of hybridization with populations of *T. amoenus*
533 and, possibly, *T. ruficaudus*. To reconstruct this complex evolutionary history, we considered
534 how incomplete lineage sorting and gene flow structured through space and time may lead to
535 contrasting patterns of shared genetic variation among populations. When we assessed the
536 placement of *cratericus* in a genus-wide context, our results suggest *cratericus* is most closely
537 related to *T. ruficaudus* albeit with low support (Figure 2). Interestingly, SVDquartets converged
538 on a different topology than both ASTRAL and BPP. ASTRAL and BPP agreed with the
539 concatenated analysis and found *cratericus* sister to *T. ruficaudus*, whereas SVDquartets
540 consistently grouped *cratericus* with *T. amoenus*. Both ASTRAL and SVDquartets appeared
541 sensitive to sampling and the branches in question received moderate to weak support across all
542 analyses (Figure 2; Supplemental Figures S3-S6). In contrast, while we found strong support
543 with BPP, these analyses were limited to a relatively small subset of data which could produce
544 misleading results given the potential for differential introgression.

545 Low branch support could be due to either a lack of information across all loci used to
546 resolve relationships (e.g., due to very short internal branches and/or homoplasy) or because
547 gene trees have independent phylogenetic histories that differ from the species tree due to
548 incomplete lineage sorting and/or hybridization. These two sources of discordance likely
549 compromise our ability to resolve this portion of the *Tamias* phylogeny; both gene concordance
550 factors (gCF) and site concordance factors (sCF) for the *cratericus*-*T. ruficaudus* split were
551 comparably low (gCF: 37.3; sCF: 38.6; Fig 2, Supplemental Figure S1).

552 One major criticism of coalescent-based summary methods, such as ASTRAL, is that
553 they may incorrectly infer a species tree because they are sensitive to stochastic variation in
554 phylogenetic signal between loci (Mirarab et al. 2016; Morales-Briones et al. 2018). However,
555 the discordance observed between our approaches likely reflect biological causes of discordance
556 such as ILS and introgression. For example, gene tree estimation error often results in a skewed
557 ratio of gCF:sCF values (Minh et al. 2020a), a pattern we do not see, even across the weakest
558 supported branches.

559 While individual tree-based approaches may be limited when local genealogies are
560 poorly resolved due to rapid divergence or limited data, our data also strongly supported the
561 conclusion that much of the phylogenetic discordance likely reflects introgressive hybridization.
562 We consistently detected genealogical asymmetry between *cratericus* lineage A and *cratericus*
563 lineage B with respect to both *T. ruficaudus* and *T. amoenus*. When we assessed the relationship
564 of either *cratericus* lineage in the absence of the other, we found strong support for two different
565 topologies. When we excluded *cratericus* lineage B, *cratericus* lineage A was consistently sister
566 to *T. amoenus*. Conversely, when we excluded *cratericus* lineage A, we recovered *cratericus*
567 lineage B as the sister group to *T. ruficaudus* (Figure 2; Supplemental Figures S8-S9). Further,

568 we inferred multiple reticulations across the *amoenus-cratericus-ruficaudus* splits, suggesting
569 there has been both recent and ancient hybridization between these lineages during their
570 diversification. Both contemporary and past hybridization events have resulted in extensive
571 shared polymorphism among the three lineages (Supplemental Figures. S16-S17) and highlight
572 how rapid diversification and introgressive hybridization can confound our ability to infer a
573 complex evolutionary history among species.

574 Integrating our phylogenetic and population genomic data, we propose a working model
575 for the evolutionary history of *cratericus* (Figure 5). There was an initial (likely geographic) split
576 between *cratericus* and other *T. ruficaudus*, followed by intermittent hybridization between
577 ancestral populations of *cratericus*, *T. ruficaudus*, and the slightly more distantly related *T.*
578 *amoenus*. Determining if the apparent genetic split within *cratericus* is associated with
579 reproductive isolation between these lineages or divergence in other morphological or ecological
580 traits awaits further sampling. While lineage A appears to be the widespread and predominant
581 form, the rarity of lineage B may be due to a lack of sampling in the presumed northern range of
582 *cratericus*. We also detected gene flow between *T. amoenus* and the more widely distributed
583 *cratericus* lineage A (Figures 3-4). While a history of recurrent hybridization likely skews
584 overall phylogenetic relationships, the sympatric population (IC) allows us to test key predictions
585 about the extent of reproductive isolation between these ecologically and phenotypically similar
586 species. In this area of sympatry, individuals clearly clustered within *cratericus* lineage A or with
587 other *T. amoenus*. Thus, species boundaries appear to be largely maintained, despite some level
588 of gene flow between *cratericus* and *T. amoenus* (Figures 3-4; Supplemental Figure S14),
589 consistent with a semipermeable species boundary (Harrison and Larson 2014). Our results also
590 indicate that *cratericus* is the predominant chipmunk species associated with xeric forests of

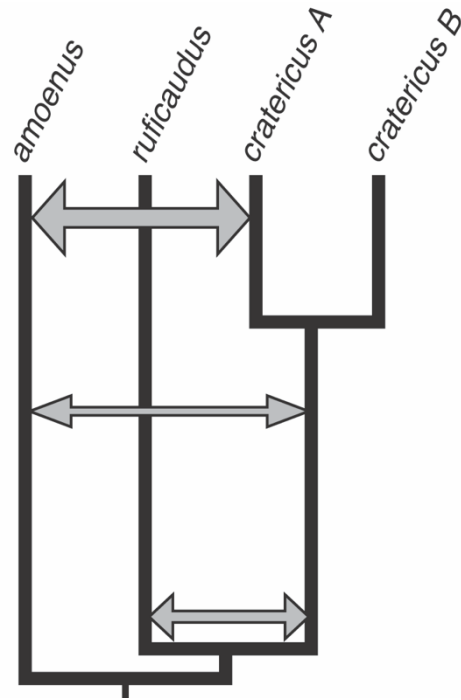


Figure 5 – Proposed species tree and evolutionary history of *T. cratericus*. *T. cratericus* has a complex evolutionary history punctuated by layered ancient and contemporary gene flow between co-distributed species. Arrow thickness indicates inferred amount of introgression.

591 south-central Idaho north of the Snake River Plain and south of the Snake River. This previously
592 unknown lineage appears to exist largely to the exclusion *T. amoenus*. Since the foundational
593 work of Grinnell a century ago (Grinnell and Storer 1924), western chipmunks have been
594 considered exemplars of ecological niche partitioning. Given the lack of obvious ecological and
595 phenotypic differentiation between *cratericus* and *T. amoenus*, sympatric populations between
596 these species may provide a compelling eco-evolutionary context to understand both the local
597 maintenance of species boundaries and niche partitioning through competitive exclusion or other
598 mechanisms.

599 Finally, our current sampling is not circumscribed by clear geographic barriers to the
600 north and west, indicating the full range of *cratericus* may be considerably larger and likely
601 extends into the remote wilderness tracks of central Idaho. Although these portions of the range

602 are more difficult to access, this diurnal cryptic species is also highly abundant and readily
603 visible around the visitor center of a National Monument that hosts ~200,000 annual visitors –
604 underscoring that cryptic biodiversity may persist even in highly conspicuous systems.

605

606 **Conflict of Interest:** The authors declare no conflict of interest.

607

608 **Data Archive:** Read sequence data are available for download at SRA under the BioProject
609 accession number XXXX.

610

611 **Author contributions:** NDH, JRD, JS, KCB, and JMG conceived and designed the study. JMG,
612 JRD, and JS acquired funding. NDH, KCB, JRD, and JMG conducted fieldwork. CMC, EN, and
613 NDH generated sequence data. NDH conducted data analyses, with guidance from BAJIS and
614 JMG. BAJIS contributed analytical tools. All authors discussed the results. NDH and JMG wrote
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