Rapid divergence and gene flow at high latitudes shape the history of Holarctic ground squirrels (*Urocitellus*)

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Abstract

Across the animal tree of life, the prevalence and evolutionary role(s) of hybridization remain incompletely understood. Rapidly radiating clades can serve as important systems for investigating these issues; however, such groups are often characterized by additional, widespread sources of gene tree discordance (e.g., incomplete lineage sorting). In this paper, we employed a multilocus dataset, Bayesian gene tree inference, and multiple species tree reconstruction methods to infer phylogeny of Holarctic ground squirrels (*Urocitellus*). We tested phylogenetic hypotheses based on previous morphological, cytological and single-locus datasets, and began to parse the causes of pervasive gene tree discordance that was observed. There is widespread incomplete lineage sorting in *Urocitellus*, consistent with rapid diversification embedded within the larger radiation of marmotine ground squirrels. We also recovered strong support for 2 instances of mitonuclear discord due to ancient hybridization among members of the

high-latitude *parryii-richardsonii-elegans* clade. These results add to a growing number of documented hybridization events in ground squirrels, suggesting their radiation is a fertile system for understanding the interplay of diversification and hybridization in animal evolution.

Keywords: incomplete lineage sorting; hybridization; mitonuclear; posterior predictive simulation; radiation; Beringia

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1. Introduction

The importance of hybridization in animal diversification has become a central issue in evolutionary biology. Long known to be a common feature of plant evolution (Arnold 2006), it is only recently that hybridization has been recognized as widespread in animals (Mallet 2005). However, a multitude of studies now demonstrate gene flow at various points along the animal speciation continuum (Hedrick 2013, Toews and Brelsford 2012). In mammals specifically, divergence in the face of gene flow has been documented across multiple taxonomic orders (e.g., Evans et al. 2001, Koepfli et al. 2015, Melo-Ferreira et al. 2012, Sullivan et al. 2014). Still, our understanding of the true prevalence of hybridization as well as its primary role(s) across the animal Tree of Life remains incomplete.

Rapidly radiating clades are ideal systems for investigating the interplay of diversification and hybridization. Some have posited that hybridization can drive radiations, occurring early in divergence and serving primarily to transfer adaptive variation (Joyce et al. 2011, Seehausen 2004), while others have viewed hybridization as a byproduct of radiation, when speciation rates simply outpace the evolution of reproductive incompatibilities (Wiens et al. 2006). However, due to short times between speciation events, rapid radiations also carry high probabilities of incomplete lineage sorting (ILS) owing to failure of gene copies to coalesce within genetic lineages (Degnan and Rosenberg 2006, Maddison 1997). Identifying hybridization in the presence of ILS is problematic because both phenomena can result in similar phylogenetic patterns (Holder et al. 2001) as well as in gene tree discordance that is difficult to parse even with large numbers of loci (e.g., Leache et al. 2014). Moreover, hybridization is often unpredictable in its' extent, being limited to organellar genome flow in some systems (e.g., Good et al. 2015, Melo-Ferreira

et al. 2011) but involving extensive nuclear gene flow in others (e.g., Cui et al. 2013, Martin et al. 2013).

Squirrels (Family Sciuridae, 58 genera) provide one example of apparently widespread hybridization in a mammal clade. In northern temperate latitudes alone, hybridization has been documented in flying squirrels (Garroway et al. 2010) and tree squirrels (Chavez et al. 2013), but most often in ground-dwelling squirrels of the tribe Marmotini (Hafner and Yates 1983, Kerhoulas et al. 2015, Nadler et al. 1971, Spiridinova et al. 2006, Thompson et al. 2015, Tsvirka et al. 2006). Marmotine ground squirrels represent a rapid, ongoing radiation (Hafner 1984, Zelditch et al. 2015) that consists of approximately 91 species in 12 genera distributed across North America and Asia (Helgen et al. 2009, Wilson and Reeder 2005). The dynamics of marmotine hybridization have been most thoroughly investigated in *Tamias* chipmunks, where mtDNA introgression has occurred repeatedly in parapatrically distributed, non-sister species across western North America (Good et al. 2008, Reid et al. 2010, Sullivan et al. 2014). Unfortunately, our understanding of core Marmotini phylogeny remains incomplete, obfuscating the extent to which similar dynamics characterize the remainder of this clade.

We reconstructed the evolutionary history of Holarctic ground squirrels (*Urocitellus*), a clade of medium-bodied, diurnal, and relatively social marmotine ground squirrels distributed across western North America, Beringia, and central Asia (Figs. 1 and 2). *Urocitellus* species have been the subject of extensive ecological, physiological and life history research over the past several decades (Barnes 1989, Boyer and Barnes 1999, Karels and Boonstra 2000, Murie and Michener 1984); still, a taxonomically comprehensive and well-resolved phylogeny of *Urocitellus* is lacking. We generated a multilocus dataset for *Urocitellus* that included all 12 species (and 33 of 36 subspecies)

and used Bayesian gene tree inference as well as 3 species tree reconstruction methods to infer phylogeny and test longstanding phylogenetic hypotheses concerning this high-latitude genus (Section 2.1). We identified gene tree discordance due to both ILS and hybridization, and used posterior predictive simulations to assess consistency of our data with the latter process. We discuss these results in the broader context of the taxonomically widespread hybridization observed to date in ground squirrels, and attempt to identify potential drivers of these phenomena.

2. Material and Methods

2.1 Phylogenetic Hypotheses

We tested 6 hierarchical phylogenetic hypotheses based on previous morphological, cytological and single-locus DNA sequence datasets. *Urocitellus* has traditionally been divided into “small-eared” and “big-eared” species groups (Figs. 1 and 2), so we first tested if these groups are reciprocally monophyletic (Table 1). The small-eared group consists of 5 small bodied, xeric adapted species distributed in and around the Great Basin, USA (*U. washingtoni*, *U. brunneus*, *U. canus*, *U. mollis*, *U. townsendii*). The big-eared group contains 7 species of larger bodied and more mesic adapted squirrels widespread in the Intermountain West, Northern Rockies, Beringia and central Asia (*U. armatus*, *U. beldingi*, *U. columbianus*, *U. elegans*, *U. richardsonii*, *U. parryii*, *U. undulatus*). We included the Idaho ground squirrel (*U. brunneus*) in the small-eared group, but note its’ previous alliance with the large-eared group based on several morphological characters (Davis 1939, Yensen and Sherman 1997).

Systematic relationships within the small-eared species group have been historically problematic. In particular, Townsend's ground squirrel (*U. townsendii*) has been considered conspecific with Merriam's ground squirrel (*U. canus*) and the Piute ground squirrel (*U. mollis*), an assemblage termed the '*townsendii* complex' (Howell 1938, Nadler et al. 1982; reviewed by Rickart 1987). These are currently considered 3 distinct species based on chromosomal variation (Cole and Wilson 2009, Nadler et al. 1982, Rickart 1985) but monophyly of this complex has not been established; thus it is unclear whether the morphological similarity of these species is due to convergence or common ancestry. We therefore tested monophyly of the *townsendii* complex (Table 1).

Previous sequence-based analyses of *Urocitellus* used a single mitochondrial (mtDNA) locus (CYTB; Harrison et al. 2003) and failed to recover reciprocally monophyletic big-eared and small-eared groups as well as a monophyletic *townsendii* complex. That analysis also recovered multiple instances of species non-monophyly. Specifically, Richardson's and Wyoming ground squirrels (*U. richardsonii* and *U. elegans*, respectively) were nested within an Arctic ground squirrel (*U. parryii*) clade, rendering *U. elegans* polyphyletic and *U. parryii* paraphyletic with respect to the former two taxa (also see Galbreath et al. 2011). Similarly, in the small-eared group, *U. mollis* was recovered as paraphyletic with respect to *U. canus* (Harrison et al. 2003). We tested the monophyly of each of these currently recognized species (*U. elegans*, *U. parryii*, *U. mollis*; Table 1) using our multilocus dataset.

2.2 Sample Selection and Sequencing

We used 2 sampling approaches based on museum specimens (Appendix A.1). The first was an expanded taxon sampling scheme, wherein 98 *Urocitellus* and 2 outgroup

marmotines (*Ammospermophilus leucurus*, *Otospermophilus beecheyi*) were sequenced for up to 2 mtDNA and 1 nuclear locus (mtDNA CYTB and control region [CTRL]; partial von Willebrand factor [VWF]). The second approach aimed to expand character sampling: 44 of these individuals (including 2 outgroups) were sequenced at 4 additional nuclear loci (breast and ovarian cancer susceptibility 1 [BRCA], beta fibrinogen [FGB], glucosidase, beta, acid [GBA], growth hormone receptor [GHR]; Table A.2). Nuclear loci were chosen based on their utility for resolving rodent relationships at a variety of temporal scales (Adkins et al. 2001, Huchon et al. 1999). Several mtDNA sequences were also obtained from GenBank (Appendix A.1).

Genomic DNA was extracted from frozen or ethanol-preserved liver or muscle tissues using Omega E.Z.N.A. extraction kits (Omega Bio-tek, Inc.) following manufacturer protocols. Ethanol-preserved samples were rinsed for 12-24 hours in ddH₂O under refrigeration prior to extraction. A number of museum skins (ventral skin clips or footpads) were also sampled for one or both mtDNA loci. These were first washed in 95% ethanol (24 hours) and then STE buffer (24 hours) under refrigeration, with regular vortexing every few hours. Extractions proceeded using the same protocols as above. All extracts were quantified on a NanoDrop 2000 (Thermo Fisher Scientific).

Standard PCR amplification took place in 25µL reactions using primer combinations given in Table A.1. All sequences were generated using Big Dye Terminator 3.1 technology (Applied Biosystems) on an ABI 3130 automated DNA sequencer in the Molecular Biology Facility in the Department of Biology at University of New Mexico. Sequences were manually edited in Sequencher v5.3 (Gene Codes Corp., Michigan, USA) and aligned using MUSCLE v3.7 (Edgar 2004) using default settings on the

CIPRES science gateway (www.phylo.org). Raw sequence assemblies were deposited in GenBank (KX278443-KX278683).

2.3 Tests of Selection

We used the software Mega v.6.06 (Tamura et al. 2013) to conduct Z-tests of hypotheses of positive selection for each exonic locus in our dataset (CYTB, VWF, BRCA, GBA, GHR; FGB is largely intronic and was omitted from tests). For this test, GBA was trimmed to include only coding regions. The test statistic (Z) was calculated as the difference between nonsynonymous and synonymous substitutions (i.e., $d_N - d_S$) divided by the square of combined variances of each type of substitution. Hypothesis tests were conducted in a pairwise manner after first binning sequences by species. The Nei-Gojobori substitution model was employed, 500 bootstrap replicates were used, and statistical significance was assessed at the $p=0.05$ level.

2.4 Phylogenetic Analysis

Gene tree inference was conducted separately on mtDNA (partitioned by locus) and single nuclear locus datasets in MrBayes v3.2.3 (Ronquist and Huelsenbeck 2003) on CIPRES. Reversible jump MCMC (Huelsenbeck et al. 2004) was used to sample model space, allowing topological inference while incorporating uncertainty in substitution parameters. A gamma distribution with four categories was used to approximate among-site rate variation. Rate priors and other parameters (except topology and branch length) were allowed to vary among mtDNA partitions; default values were used for other priors in all analyses. Times to convergence varied significantly, with MCMC runs of 25 million (mtDNA), 50 million (BRCA, FGB, GHR), or 150 million (GBA, VWF) generations. Proper

207 mixing was assessed by viewing trace plots in Tracer v1.6.0 (Rambaut et al. 2014), and
208 we required effective sample sizes >200 for all estimated parameters. Posterior
209 distributions of gene trees were summarized either as maximum clade credibility (MCC)
210 phylograms (in text) or majority-rule consensus phylograms (Fig. A.1) following removal
211 of a burn-in of the first 20% of trees using TreeAnnotator v1.8.2.

212
213 Species trees were inferred from the expanded character dataset (42 *Urocitellus* +
214 outgroups) using 3 different methods. First, we used *BEAST v1.8.1 (Drummond et al.
215 2012), which simultaneously infers gene trees and their encasing species tree in a
216 hierarchical coalescent framework. *BEAST has been shown to have power in species
217 tree inference even if ILS exists (e.g., Lanier et al. 2014). However, *BEAST does not
218 model horizontal transfer as a source of gene tree discord, and results could be
219 misleading if horizontal transfer goes undetected. *BEAST analyses were conducted
220 using all loci and nuclear loci only (5), both via the CIPRES gateway. Analyses were run
221 for 750 million generations, sampling posterior distributions every 2500 generations and
222 assessing proper mixing in Tracer as described above. A burn-in of 50% of trees was
223 removed and posterior distributions were summarized as MCCs as above.

224
225 The software STAR (Liu et al. 2009) and MP-EST (Liu et al. 2010) were also used to
226 infer species trees from the expanded character dataset. Inputs for both are gene trees
227 (which are assumed to be known without error). Of the variety of summary species tree
228 methods recently developed, 2 broad categories exist: those that use only gene tree
229 topologies and those that also use the coalescence times inferred on gene trees
230 (DeGiorgio and Degnan 2014). STAR and MP-EST are in the former category (i.e., use
231 topologies). Briefly, STAR estimates species trees based on a distance matrix describing
232 the average rank coalescences of all possible taxon pairs (Liu et al. 2009), while MP-

EST estimates species trees by maximizing a pseudo-likelihood function which takes into account all possible rooted taxon triples in gene trees that could be encoded by a species tree (Liu et al. 2010).

We chose STAR and MP-EST for 2 reasons. First, because they do not model ploidy, the influence of mtDNA relative to nuclear loci is reduced. It has been shown that mtDNA can have disproportionately strong influence on species trees in *BEAST due to its high variability and because assumptions of lower ploidy (which is modeled by *BEAST) are not always met (Jockusch et al. 2015). This is especially true in organisms with female-biased sex ratios and/or high male migration, such as occurs in *Uroditellus* (Byrom and Krebs 1999, Karels and Boonstra 2000). Second, and relatedly, we used these methods because both may be sensitive to possible instances of mtDNA introgression, which has been commonly documented in ground squirrels. We note that introgression violates the assumptions of all 3 methods used here; however, confident identification of introgressed loci is not always possible at shallow phylogenetic levels or when ILS is pervasive. STAR and MP-EST may therefore decrease the effects of such events if they have occurred (Liu et al. 2010).

Most coalescent-based summary methods (including STAR and MP-EST) assume gene trees are known without error, which is rarely the case empirically. Bootstrapping approaches aim to incorporate gene tree uncertainty into species tree estimates (Seo 2008); however, using the uncertainty from Bayesian posterior distributions, where gene trees are represented in approximate proportion to their likelihood, is a better approach (Liu et al. 2009). This method may be intractable with very large numbers of loci (due to time required for Bayesian gene tree analysis), but it is a reasonable alternative to bootstrapping using smaller numbers of loci. To accomplish this, we used custom scripts

in R (R Core Team 2015) to randomly sample 1000 trees from each gene tree posterior from MrBayes runs after removing 20% burn-in. We ran both STAR and MP-EST for 1000 iterations, each time drawing a single tree from each of the randomly downsampled gene tree distributions without replacement (thus inputs for every iteration of the species tree reconstruction methods contained 6 randomly sampled gene trees, 1 per locus). We summarized the results of both analyses (total of 1000 species tree reconstructions each) as MCC cladograms in TreeAnnotator, and performed the routine 3 separate times to ensure congruence.

2.5 Multidimensional Scaling

To compare and visualize variation in individual gene tree distributions, we used the Tree Set Visualization package (Tree Set Vis; Hillis et al. 2005) in Mesquite v3.03 (Maddison and Maddison 2015). Tree Set Vis performs multidimensional scaling on a pairwise matrix of Robinson-Foulds distances among topologies. Analyses were conducted on 500 trees selected randomly from the 6 MrBayes posterior distributions (mtDNA + nuclear loci; $N=3000$ trees total), and all were run until the stress function stabilized. We repeated each analysis 3 times to ensure congruence.

2.6 Haplotype Network

Upon identifying possible instances of mitonuclear discord in *Urocitellus*, we assembled an extended CYTB dataset based the dataset generated in this study combined with additional published and unpublished sequences (Appendix A.1). We did this to visualize in greater detail the distribution of haplotypic diversity for all subspecies of *U. parryii*, *U. richardsonii* and *U. elegans*, as well as just those taxa involved in putative hybridization

events (i.e., *U. elegans elegans*, *U. richardsonii*, *U. parryii parryii*, and *U. p. kennicottii*).

We computed both haplotype networks using the package ape (Paradis et al. 2013) in R.

Default settings were used for all parameters; the default distance parameter is the

uncorrected distance among sequences under an infinite-sites model.

2.7 Hypothesis Tests

We tested the 6 phylogenetic hypothesis referenced above (Section 2.1, Table 1) using

nodal support values as a significance criterion. We considered nodal support (posterior

probabilities [PP], or “bootstrap” values in the case of STAR and MP-EST) greater than

or equal to 0.95 to constitute support for a particular hypothesis; positive or negative

results of all tests are reported relative to this threshold.

However, there are two potential issues with this approach. First, although 0.95 PP is

commonly considered high nodal support, PP is not directly interpretable in a frequentist

framework (i.e., recovering a clade with 0.95 PP support is not statistically equivalent to

rejecting a hypothesis of non-monophyly under a p-value of 0.05). Moreover, this

approach can lead to inflated Type I error rates (Alfaro and Holder 2006). We maintain

the PP=0.95 threshold but also refer readers to actual PP or nodal support values.

Second, PP from *BEAST and support values from our STAR and MP-EST bootstrap

procedure are not directly comparable. The same would apply to comparisons of PP and

traditional (nucleotide site) bootstrapping, although studies have shown that bootstrap

proportions and PP sometimes have relatively high correlation (Alfaro et al. 2003).

However, we note that support values for STAR/MP-EST summary trees provide a

measure of clade support that incorporates uncertainty in gene tree distributions and

model parameters, and thus remain more directly interpretable than support values generated using nucleotide and/or locus bootstrapping.

2.8 Posterior Predictive Checking

Following identification of putative mitonuclear discord in *Urocitellus*, we assessed this possibility more rigorously using the posterior predictive method of Joly et al. (2009). That method (implemented as JML; Joly 2012) compares genetic distances observed between taxon pairs with the minimum genetic distances found from simulation on a set of species trees, the latter of which have been inferred under the coalescent with no migration (e.g., in *BEAST). If empirical genetic distances are significantly lower than those from simulations (i.e., if they are lower than expected based on the time since speciation and given ILS), a hypothesis of discordance due to ILS is rejected, supporting hybridization as a potential cause of gene tree discordance.

We performed posterior predictive checks using the CYTB expanded taxon dataset described previously (Section 2.2). Although the CTRL dataset should also encode a signature of mtDNA introgression, it has been shown that JML has reduced power to distinguish hybridization from ILS as sequence length decreases (Joly et al. 2009). Given that property, we also removed 4 samples from the CYTB dataset with only partial sequences (representing *U. canus canus* and *U. c. vigilis*). We used species tree files generated by *BEAST from the expanded character dataset (mtDNA + nuclear) as input for JML, removing a burn-in of 50% of trees as described previously. We employed a heredity scalar of 0.5, a GTR model of sequence evolution, and mean values for sequence parameters (base frequencies, rate matrix, and mutation rate) extracted from original *BEAST .log files. We ran a second JML analysis using median values for the

above parameters to ensure consistency. Default values were used for all other input parameters. We ran simulations on 10000 species trees and assessed significance at the $p=0.1$ level (the default setting).

3. Results

3.1 Gene Tree Concordance and Discordance in *Uroditellus*

The partitioned mtDNA dataset with extended taxon sampling yielded a robustly supported tree (Fig. 3). The majority of nodes received >0.95 PP, including all basal nodes except 3 (i.e., those depicting interspecies relationships), which represents an improvement on single mtDNA gene analyses (Harrison et al. 2003). Monophyly of the big-eared group is strongly supported while monophyly of the small-eared group is not (Table 1), and the latter is true whether or not *U. brunneus* is included in the small-eared group. The mtDNA topology also failed to support monophyly of the *townsendii* complex. All 3 instances of species mtDNA non-monophyly encountered previously (Galbreath et al. 2011, Harrison et al. 2003) were also recovered in our tree (Fig. 3, Table 1). Specifically, *U. mollis* is recovered as paraphyletic with respect to *U. canus*, and *U. richardsonii* and *U. elegans elegans* are both nested within an Arctic ground squirrel (*U. parryi*) clade, rendering the latter 2 taxa non-monophyletic. We note that the 2 remaining subspecies of *U. elegans* (*U. e. aureus* + *U. e. nevadensis*) are not nested within *U. parryi*, but positioned basal to it.

The VWF tree with expanded taxon sampling failed to support a big-eared clade or a small-eared clade at 0.95 PP (Fig. 4, Table 1); however, both groups were recovered with >0.80 PP. Support for a monophyletic *townsendii* complex was poor, with strong

support (>0.95 PP) instead obtained for the inclusion of *U. washingtoni* within this group.

As expected, the VWF tree differs from mtDNA in overall resolution; 7 of 12 *Urocitellus* species are recovered as non-monophyletic in VWF. Interestingly, however, *U. parryii* and *U. elegans* are not among them, although *U. mollis* is (Table 1).

VWF and the 4 additional nuclear loci analyzed herein had distinct mutational profiles, including differences in mutation rate and patterns of among-site variation (Table A.2). Still, no nuclear locus strongly supported monophyly of big-eared or small-eared groups or of the *townsendii* complex (Table 1, Fig. A.1). Multidimensional scaling of Robinson-Foulds distances in Tree Set Vis revealed that posterior distributions of inferred gene trees (mtDNA + 5 nuclear) were completely non-overlapping in tree space (Fig. 5). This inability of multiple independent markers to track deep divergences is consistent with ILS as a result of rapid diversification, which also characterizes the tribe Marmotini as a whole (Hafner 1984, Zelditch et al. 2015). In such situations, many loci are expected to have low probabilities of coalescence between speciation events (Degnan and Rosenberg 2006).

Conversely, single nuclear loci appeared to support robust tests of the 3 hypotheses of species monophyly (*U. elegans*, *U. parryii*, *U. mollis*). This is not surprising because, if radiations are old enough, sufficient variation may be accumulated to discriminate species despite the fact that ILS obscures deeper relationships. Monophyly of *U. parryii* and *U. elegans* was supported by 4 of 5 nuclear loci each (Table 1, Fig. A.1), providing strong evidence that both are valid species, concordant with morphological and cytological assessments as well as current taxonomic arrangements (Howell 1938, Nadler et al. 1984, Robinson and Hoffmann 1975). However, unlike in *U. parryii* and *U. elegans*, no nuclear loci support *U. mollis* monophyly (Table 1, Figs. 4 and A.1).

All tests of positive selection in coding loci (CYTB, VWF, BRCA, GBA, GHR) were negative at the $p=0.05$ level (Appendix A.2). Thus, recent episodes of selection are not supported as contributors to gene tree discordance among these particular loci.

3.2 Species Tree Reconstructions

Despite low phylogenetic resolution in individual loci, we recovered stronger support for interspecies relationships using 3 species tree reconstruction methods based on the expanded character dataset. When all loci (mtDNA + nuclear) were included, monophyly of a big-eared group was consistently supported at 0.95 PP, whereas monophyly of a small-eared group was supported only in *BEAST analyses (Fig. 6, Table 1). Support for monophyly of the *townsendii* complex was supported by 2 of 3 methods (*BEAST and STAR). Conversely, when only nuclear loci were included, species trees failed to support any of these hypotheses at the 0.95 level except for in one instance (small-eared monophyly was supported by *BEAST). Nevertheless, we note that monophyly of the big-eared group, small-eared group, and *townsendii* complex as traditionally defined were each supported at >0.75 across methods and datasets (Fig. 6).

We found general topological correspondence among species trees and datasets, despite some variation in nodal support (Fig. 6). Inclusion of mtDNA increased support for hypotheses across species tree reconstruction methods, and impacted topology only minimally. We initially predicted that effects of mtDNA inclusion would be more significant, as the mtDNA tree differs from nuclear gene trees and the former marker can have disproportionate influence in *BEAST due to higher variability and/or violated assumptions related to its lower ploidy (e.g., Jockusch et al. 2015). On the contrary,

*BEAST trees using all loci versus nuclear loci were topologically identical and, although nodal support differed, there were no consistent trends in these differences. Conversely, STAR and MP-EST produced topologies that differed by 1 branch each among datasets (*U. undulatus* in STAR, *U. mollis* in MP-EST), suggesting greater but still subtle influence of mtDNA in those methods.

Relationships among small-eared species were generally better resolved than among big-eared species (Fig. 6). Poor support for big-eared species relationships is driven by instability in placement of *U. armatus*, *U. beldingi*, and *U. columbianus*. A sister relationship of *U. armatus* and *U. beldingi* (parapatric species in western North America; Fig. 1) was strongly supported by mtDNA (Fig. 3) but not recovered in any species tree. Instead, the most common (but weakly supported) relationship was *U. beldingi* as sister to *U. columbianus*. The Columbian ground squirrel (*U. columbianus*) has sometimes been considered sister to the Arctic ground squirrel (*U. parryii*) based on morphological (Howell 1938) and chromosomal (Nadler 1966, Nadler et al. 1974, Nadler et al. 1984) data, but we found no support for that arrangement, consistent with previous work (Harrison et al. 2003, Robinson and Hoffmann 1975). Finally, placement of the long-tailed ground squirrel (*U. undulatus*) is inconsistent; this species is exclusively Palearctic in distribution and may represent a long branch that is difficult to place phylogenetically.

3.3 Support for Hybridization

Based on phylogenetic inferences (summarized in Table 1), we hypothesize that mitonuclear discord in the *parryii-richardsonii-elegans* (PRE) clade is the result of hybridization leading to mtDNA capture. This is based on the fact that a majority of nuclear loci support monophyly of *U. elegans* and *U. parryii*, and lineage sorting in

mtDNA is expected to occur faster than in nuclear loci based on lower ploidy and higher mutation rate (e.g., Table A.2). However, a single hybridization event between *U. parryii* and the most recent common ancestor of *U. richardsonii* and *U. elegans* (likely sister species, see 3.2 above) is unsupported because remaining subspecies of *U. elegans* (*U. e. aureus* + *U. e. nevadensis*) carry ancestral, non-introgressed haplotypes (Fig. 3). We therefore propose that 2 ancient, sequential hybridization events occurred in the *PRE* clade, first from high arctic populations of *U. parryii* (*U. p. kennicottii* or *U. p. parryii*) to a single subspecies of *U. elegans* (*U. e. elegans*), and second with the latter taxon acting as a mitochondrial vector, transmitting a recently acquired *U. parryii* mtDNA genome to its sister species *U. richardsonii*.

This sequential hybridization scenario is supported by the CYTB haplotype network (Fig. 7), calculated using specimens of the 4 relevant taxa (Table A.1), as well as by genetic distances between those taxa returned from JML analyses (Table 2, and below). However, distances between *U. parryii*-*U. e. elegans* and *U. parryii*-*U. richardsonii* differ only minimally, and the directionality of gene flow we propose must be interpreted with caution until additional nuclear data are available to resolve species phylogeny. The haplotype network for all *PRE* taxa is also ambiguous with respect to the most parsimonious scenario of mtDNA gene flow (Fig. A.2). Thus, we cannot at present reject an alternate scenario of introgression (*U. parryii* -> *U. richardsonii* -> *U. elegans*), nor the possibility that this phylogroup carries an ancestral *U. richardsonii* mtDNA genome, such that *U. richardsonii* hybridized with *U. parryii* (ancient) and *U. elegans* (more recently).

Results of posterior predictive checking in JML support the assertion that mitonuclear discordance in *Urocitellus* is due to a process other than ILS. JML runs recovered 5 interspecific comparisons (of 91 total) with observed mtDNA distances significantly lower

than expected under a coalescent model (Table 2). Notably, these include all 3 pairwise species comparisons in the *PRE* clade. Inspection of JML output confirmed that these results are driven solely by comparisons among *U. richardsonii*, *U. parryii parryii*, *U. parryii kennicottii*, and *U. elegans elegans*, consistent with the hybridization scenario we outline above. Table 2 also lists the number of significant comparisons at the subspecific level as a percentage of all such possible comparisons, highlighting the taxonomically isolated nature of introgression.

In addition to the *PRE* clade, JML recovered comparisons between *U. beldingi* and each of *U. mollis* and *U. canus* as deviating from a strict coalescent model. This is interesting because no evidence for mtDNA introgression exists in these taxa (Fig. 3), and minimum genetic distances are much higher than within the *PRE* clade (Table 2). Moreover, no comparisons were significant at the $p=0.05$ level, which possibly is reflective of power issues. We return to both of these points in the Discussion.

3.4 New phylogeographic insights in the Arctic ground squirrel

The expanded taxon mtDNA dataset included complete taxon sampling at the subspecific level for *U. parryii*, including 2 subspecies never before sequenced (*U. p. parryii* and *U. p. stegnejeri*). We recovered 4 major mtDNA clades as observed by previous workers (Eddingsaas et al. 2004, Galbreath et al. 2011; Figs. 3 and 8): Arctic, Southeast (SE Alaska, NW Canada), Southwest (SW Alaska, including Alaskan Peninsula and Aleutian Arc), and Beringian (Siberia, Seward Peninsula, interior Alaska). Our reconstructions placed *U. p. parryii* within the Arctic clade, sister to *U. p. kennicottii*, and placed *U. p. stegnejeri* (Kamchatka, Russia) in the Southwest clade. These results

demonstrate that 2 distinct *U. parryii* mtDNA clades currently have amphiberian distributions.

4. Discussion

4.1 The Complex Genomic History of *Urocitellus*

To date, a comprehensive and well-resolved phylogeny of *Urocitellus* has proven elusive. This is despite extensive research using chromosomal and karyotypic (Nadler 1966, Nadler et al. 1971, Nadler et al. 1984, Rickart 1985), protein electrophoretic (Nadler and Hoffmann 1977, Nadler et al. 1974) and morphological (Robinson and Hoffmann 1975) data. While these previous studies greatly clarified issues of systematics and biogeography, the lack of a model-based phylogenetic framework for analyzing these data, incomplete taxon sampling, and a paucity of molecular research in intervening years have left important questions unanswered (e.g., Table 1). We addressed this using the most taxonomically complete sequence dataset available for *Urocitellus*, evaluating mtDNA and nuclear loci independently, and utilizing 3 methodologically distinct species tree methods that should also differ in their sensitivity to mtDNA inclusion as well as to isolated introgression events.

A crucial first step in understanding *Urocitellus* diversification lies in fully resolving the phylogenetic status of big-eared and small-eared groups. Small-eared *Urocitellus* are small-bodied, adapted to life in arid environments and geographically narrowly restricted to the Great Basin and surrounding regions of western North America (Fig. 2). In contrast, big-eared species are up to an order of magnitude larger in size (in *U. parryii*), more mesic-adapted, occupy larger geographic ranges, and have a much wider

latitudinal distribution that also spans multiple continents (Fig. 1). Some big-eared species also display extreme physiological adaptations for hibernation (Barnes 1989, Boyer and Barnes 1999). Reciprocal monophyly of these groups would therefore presumably represent an important adaptive dichotomy within the genus.

All species tree reconstruction methods (*BEAST, STAR, MP-EST) indicate this dichotomy exists, but support for monophyly of the groups varies from moderate to very high depending on the dataset and species tree reconstruction method. We also recovered moderate to high support (0.79-0.99 nodal support; Fig. 6) for monophyly of a 'townsendii complex' within the small-eared group. These small-eared taxa are similar in morphology and protein electrophoretic characters (Nadler et al. 1974), but divergent in karyotype (2N=36-46; Nadler 1966, Rickart 1985). Thus, our results suggest that these latter taxa are sister species that have undergone instances of significant chromosomal evolution in spite of their morphological conservatism.

We submit that our inability to accept the phylogenetic hypotheses above is due to pervasive ILS owing to rapid diversification of *Urocitellus*, which itself is reflective of the larger radiation of marmotine ground squirrels. That scenario is supported by the poor resolution within, as well as topological disparity among, gene trees (Figs. 5 and A.1), each of which point to ILS concentrated deep in *Urocitellus* phylogeny. While this resulted in negative tests for many hypotheses, we note that such results may not be consistent with formal rejection of phylogenetic hypotheses. Instead, failure to obtain robust clade-level support may be due to low statistical power, directly resulting from insufficient information in nuclear loci. Nevertheless, such inconclusive results were surprising given the amount of data available (5270bp), variability present in locus-specific mutational profiles (Table A.2), and evidence from previous studies

demonstrating the power of the coalescent function in *BEAST (Lanier et al. 2014) and the performance of STAR and MP-EST (Degiorgio and Degnan 2014) in spite of moderate ILS. In the future, higher statistical power may be found with genome-scale sampling of *Urocitellus*.

4.2 Sources of mtDNA Non-Monophyly in *Urocitellus*

ILS, hybridization and incorrect species delimitation are 3 common explanations for non-monophyly in gene trees of vertebrate species (McKay and Zink 2010). While hybridization and ILS result in true non-monophyly of gene copies, incorrect species delimitation can lead to discordance between gene trees and recognized taxonomic boundaries (McKay and Zink 2010). As we discuss, ILS is apparent at the interspecific level in *Urocitellus*, particularly in nuclear loci. Yet, despite this, species delimitation remains possible in many cases (Table 1 and Fig. A.1). Our taxonomically comprehensive multilocus dataset therefore allowed robust evaluation of current systematic and taxonomic arrangements at and below the species level; this includes the latter 3 phylogenetic hypotheses outlined above (i.e., monophyly of *U. parryii*, *U. elegans* and *U. mollis*; Table 1).

Phylogenetic inferences, as well as posterior predictive simulations, support hybridization leading to introgression as the source of mtDNA non-monophyly in *U. parryii* and *U. elegans*. We hypothesize that mtDNA gene flow occurred twice: first, from the Arctic clade of *U. parryii* (*U. p. kennicottii* or *U. p. parryii*) to the subspecies *U. e. elegans*, and second, from *U. e. elegans* to *U. richardsonii* (Fig. 8). Our study demonstrates the importance of a posterior predictive approach in particular when attempting to distinguish hybridization from ILS as explaining mitonuclear discordance.

While we were unable to reject a role for ILS at the $p=0.05$ level, we note that the statistical power of the JML approach is sensitive to misspecification of substitution model parameters, length of the locus under investigation, and the time elapsed between events of speciation and hybridization (Joly et al. 2009). Thus, close relationship of members of the *PRE* clade as well as the small length of CYTB (1140bp) could explain why we attained only marginal statistical significance in rejecting ILS in this instance. Additional mtDNA data (to increase sequence length) as well as nuclear data (to further increase precision of species tree estimates) would be useful to further test our proposed hybridization hypothesis.

Conversely, there is no support for either ILS or hybridization as a driver of mtDNA non-monophyly in *U. mollis*. ILS is unlikely due to the expected faster sorting of mtDNA relative to nuclear loci, coupled with the fact that nuclear loci often delimit other *Urocitellus* species (but never *U. mollis*; Table 1). Hybridization (with *U. canus*) was also unsupported by JML, despite the performance of the method for closely related species in the *PRE* clade (we note that *U. c. canus* was not included in JML runs, however). We therefore hypothesize that current taxonomy inaccurately reflects species boundaries in this taxon. Indeed, multiple species were originally described from within *U. mollis* (e.g., Merriam 1913), and electrophoretic differences were reported among subspecies (Nadler et al. 1974). Expanded sampling is therefore warranted for accurate species delimitation in *U. mollis*. Future work should focus on range-wide sampling, especially along the Snake River, Idaho, where ranges of subspecies *U. m. artemesiaae* and *U. m. idahoensis* abut that of *U. m. mollis* (Fig. 2).

Finally, it is interesting that posterior predictive checking rejected a role for ILS in explaining patterns of mtDNA genetic distance between *U. beldingi* and *U. mollis* (the *U.*

beldingi - *U. canus* comparison was also significant at the $p=0.1$ level; Table 2). There is no evidence for hybridization among these taxa in mtDNA. Instead of hybridization, our results may result from the large discrepancies in *U. beldingi* placement within the big-eared clade existing between mtDNA and species trees (Figs. 3 and 6). Specifically, uncertainty in *BEAST species tree posterior distributions could lead to an inflation of simulated mtDNA distances between *U. beldingi* and small-eared taxa such as *U. mollis* relative to that observed in empirical data. Such a scenario could result in increased susceptibility of the posterior predictive approach to Type I error in cases where species trees are poorly resolved, but mtDNA (or other single locus) trees display high and conflicting support. Application of the method to similar situations in additional clades is therefore warranted.

4.3 The Biogeography of Diversification in *Urocitellus*

Differences in the distribution and habitat preferences of small-eared and big-eared *Urocitellus* have long been recognized (Howell 1938, Davis 1939, Durrant and Hansen 1954), but new insights as well as questions emerge when viewed in light of our results. A clear feature of *Urocitellus* evolution is colonization of high latitudes and altitudes by members of the big-eared group (Fig. 1). Four of these species inhabit latitudes higher than 50°N: *U. columbianus* (northern Rocky Mountains), *U. richardsonii* (northern Great Plains), *U. parryii* (North American high arctic and Siberia), and *U. undulatus* (central Asia and Siberia). Our results suggest multiple high latitude colonizations occurred in the history of the genus, which is notable given the demands of arctic and subarctic existence on homeothermic animals. However, the number and timing of arctic colonizations in particular remains uncertain. The exclusively Palearctic *U. undulatus* must have colonized Asia via Beringia just as *U. parryii* has done more recently

(Galbreath et al. 2011); if *U. undulatus* shares a most recent common ancestor with the *PRE* clade (as suggested by mtDNA, *BEAST and STAR analyses), then colonization of arctic habitats must have occurred only once. However, additional data are needed to rule out an alternative scenario of 2 distinct arctic colonization events.

The concept of high-latitude diversification and adaptation is perhaps best embodied by the Arctic ground squirrel (*U. parryi*), which is the northernmost species of squirrel in the world. Intraspecific diversification of *U. parryi* within Beringia has been strongly shaped by Quaternary climate change (Eddingsaas et al. 2004, Galbreath et al. 2011, Nadler and Hoffmann 1977), and our data shed additional light on that history. First, we show that the Arctic clade of *U. parryi* is widespread across the North American high arctic (Fig. 8), such that it likely expanded from a Nearctic glacial refugium following the Last Glacial Maximum. We also demonstrate the occurrence of 2 separate mtDNA clades in core Beringia during the late Pleistocene, each of which currently persists in North American and Asian refugia. Based on that result, these clades (Beringia and Southwest [Alaska] clades *sensu* Cook et al. 2010, Eddingsaas et al. 2004, Galbreath et al. 2011) are best considered the “Northern Beringia” and “Southern Beringia” clades (Fig. 8; for the purposes of this study, we include the Southeast clade of previous authors in the “Northern Beringia” clade). The alliance of *U. p. stejnegeri* with the southwestern Alaska mtDNA clade (*U. p. ablusus*, *U. p. kodiacensis*, *U. p. nebulicola*) to comprise the Southern Beringia clade reveals a previously unrecognized level of mtDNA diversity in the Palearctic, and we note that this fails to support recent taxonomic treatments subsuming all Palearctic *U. parryi* into a single subspecies (*U. p. leucostictus*; Kryštufek and Vohralík 2013).

The phylogeographic patterns discussed above are intriguing because late Pleistocene

Beringia is often reconstructed as an open steppe environment. However, Guthrie (2001) proposed a Beringian “mesic buckle” as an ecological barrier for some Pleistocene fauna, comprised of mesic tundra or forest and stretching north-south in the region of the modern-day Bering Strait. If inhospitable enough, this or other features may have fostered the isolation of *U. parryii* mtDNA clades in core Beringia, even if they were somewhat porous in space or time. In contrast, nuclear data have been interpreted as telling a story of high migration between the Northern Beringia and Southern Beringia clades (Galbreath et al. 2011). Those authors hypothesized that the significant structuring of mtDNA relative to nuclear loci could be driven by female philopatry and highly male-biased dispersal (Byrom and Krebs 1999, Karels and Boonstra 2000), which our data further support. In the future, it will be important to investigate whether a narrative of elevated mtDNA diversification, driven by demographic biases and superimposed on contrasting nuclear backgrounds, characterizes additional *Uroditellus* across the Holarctic.

The biogeographic patterns we outline for the big-eared group stand in contrast to those of small-eared *Uroditellus*, which are geographically limited to areas in and surrounding the Great Basin. Range sizes of small-eared taxa are also relatively small (except *U. m. mollis*). However, to date, the processes that have promoted diversification of low-elevation taxa (such as *Uroditellus*) in this region remain poorly understood (Riddle et al. 2014). Figure 2 highlights the geographic apportionment of diversity in small-eared *Uroditellus*, which appears demarcated in several instances by major river drainages. We therefore consider it possible that the evolutionary narrative of small-eared taxa is one of long-term endemism and *in situ* allopatric diversification within the Great Basin shrub-steppe ecosystem, potentially induced by shifts in the presence and location of

glacial lakes and ancient/modern river systems. Testing this hypothesis will be possible with ongoing geographic sampling and taxonomic reconsideration of the group.

Finally, full resolution of *Urocitellus* phylogeny and completion of species-level phylogeographies will be paramount for effective management of imperiled taxa. This is particularly true for small-eared species, whose disproportionate representation on conservation lists is at least partly related to their restricted geographic distribution. Under the U.S. Endangered Species Act, 1 subspecies (*U. brunneus brunneus*) is currently listed as Threatened, 1 species (*U. washingtoni*) is listed as Candidate, and another species is Of Concern (*U. townsendii*). Anecdotal evidence for population-level declines also exists in *U. canus* (Yensen and Sherman 2003). Perhaps the most pressing result of our analysis is recovery of the Idaho ground squirrel (*U. brunneus*) as basal to the remainder of small-eared species, a relationship not supported by mtDNA or even most nuclear genes individually (Figs. 3 and A.1; Harrison et al. 2003). *U. brunneus* has the most restricted geographic distribution of any *Urocitellus* (5 counties in western Idaho; Fig. 2), and our data suggest the possibility that it is an ancient and divergent lineage. This places renewed importance on continued monitoring of natural and anthropogenic threats to this taxon.

4.4 What Drives Hybridization in Marmotini?

Rapidly radiating clades are ideal systems for examining many evolutionary phenomena, including the interaction of hybridization and diversification. Maddison (1997) and others have suggested that phylogeny is not only a depiction of historical splitting events, but also a history of changing probabilities of interbreeding. Mallet (2005) calculated that roughly 10% of animal species hybridize, and it is now apparent that gene flow can

proceed at various points along the speciation continuum without interrupting or reversing speciation itself (e.g., Evans et al. 2001, Koepfli et al. 2015, Melo-Ferreira et al. 2012, Sullivan et al. 2014). Yet some clades appear more prone to hybridization than others, and conclusive examples of hybridization now exist in at least 6 of 11 recognized marmotine genera containing >1 species (Hafner and Yates 1983, Kerhoulas et al. 2015, Nadler et al. 1971, Spiridinova et al. 2006, Sullivan et al. 2014, Thompson et al. 2015).

But what are the factors facilitating such widespread gene flow?

We identify 3 potential drivers of hybridization in *Urocitellus* that are not mutually exclusive and may also extend to other ground squirrel genera. First, squirrels in general and ground squirrels in particular are morphologically conserved (Casanovas-Vilar and van Dam 2013, Emry and Thorington 1984, Zelditch et al. 2015). Morphological diversification in *Urocitellus* has been reconstructed as among the lowest of all marmotine clades (Zelditch et al. 2015), and a significant amount of standing morphological variation in this genus may simply be due to allometry (Pearson 1981, Robinson and Hoffmann 1975, BSM unpublished data). A similar pattern of conservatism in those morphological traits directly involved in prezygotic incompatibility could facilitate widespread hybridization.

Second, conservatism in ecologically relevant traits, such as phenology and habitat preference, could also drive hybridization. There is overlap in both of these traits within each of the small-eared and big-eared groups of *Urocitellus*. For example, most big-eared species occur in parapatry across mesic and semi-arid grasslands of western North America (Fig. 1). They are known to partition habitat in zones of sympatry, but not in allopatry, suggesting a lack of ecological differentiation (Durrant and Hansen 1954). Additionally, habitat partitioning in sympatry can be incomplete, resulting in existence of

species pairs in syntopy (Davis 1939, Durrant and Hansen 1954, McLean personal observation). Such a lack of differentiation could facilitate hybridization if other major isolating mechanisms are absent, similar to what has been documented in non-sister species of *Tamias* chipmunks (Good et al. 2008, Reid et al. 2010, Sullivan et al. 2014).

Third, Quaternary climate change has repeatedly reorganized faunal and floral communities, creating significant opportunities for secondary contact and gene flow. This is particularly true at high northern latitudes (Hewitt 2000, Lessa et al. 2003), which are themselves emerging as prime venues for investigating the interplay of rodent diversification and hybridization (Chavez et al. 2014, Kerhoulas et al. 2015, Runck et al. 2009). The power of past climate change to create unique and unpredictable opportunities for gene flow is evidenced in the *PRE* clade; not only do all 3 taxa now occur in allopatry, but the directionality of gene flow we propose is unparsimonious with reference to current distributions. First, hybridization occurred between *U. parryii* and *U. e. elegans* despite the fact that the range of *U. richardsonii* currently lies between them. Second, according to our proposed scenario, it was the Arctic clade of *U. parryii* (not the geographically proximate *U. p. plesius*) that came into contact with *U. e. elegans*. Third, secondary introgression occurred between *U. e. elegans* and *U. richardsonii* despite the fact that *U. richardsonii* is currently sympatric only with *U. e. aureus*, with which it is documented to hybridize (Nadler et al. 1971) but has not shared mtDNA. Thus, while the lack of morphological or ecological isolating mechanisms sets a biological “stage” for hybridization, climatic and geographic contingencies can act as potent drivers of this process. A rigorous historical perspective will be crucial to future assessments of hybridization dynamics in other northern taxa.

4.4 The Possibility of Adaptive Gene Flow in *Urocitellus*

747

748 The ultimate role of hybridization in animal diversification remains incompletely
749 understood (but see Hedrick 2013). Wiens et al. (2006) suggested that, in rapidly
750 diversifying clades, hybridization might be a consequence of radiation, with speciation
751 outpacing the evolution of reproductive incompatibilities. Seehausen (2004) suggested
752 that hybridization might also drive radiations through transfer of adaptive variation. Both
753 models accommodate hybridization as adaptive, but that of Seehausen (2004) posits a
754 causal role for adaptive introgression in radiations.

755

756 Many authors have ascribed instances of mtDNA introgression to adaptive advantage of
757 mtDNA haplotypes (Toews and Brelsford 2012), but evidence for this is slim, largely
758 because it requires linking mtDNA functionality to individual fitness consequences (Storz
759 and Wheat 2010). However, evidence is mounting that mtDNA variation does underlie
760 important respiration-related adaptations (Shen et al. 2010, Scott et al. 2015, Toews et
761 al. 2013, Wilson et al. 2013), and that differential fitness consequences can also result
762 from interactions of mtDNA superimposed on different nuclear backgrounds (Ellison and
763 Burton 2006, Hill et al. 2015, Levin et al. 2014).

764

765 Was hybridization adaptive in *Urocitellus*? Or was this a nonadaptive result of secondary
766 contact between reproductively compatible species, driven by Quaternary climate cycling
767 and/or demographic change? All *Urocitellus* are obligate hibernators (Thorington et al.
768 2012), but *U. parryii* is considered an extreme hibernator, capable of enduring body
769 temperatures below 0° C for several weeks (Barnes 1989, Buck and Barnes 1999).
770 Mitochondria are main sites for cellular energy production and play crucial roles during
771 hibernation and intermittent arousals in mammals, such as maintaining body
772 temperatures above subzero arctic soil temperatures and functioning in non-shivering

thermogenesis (Staples 2014, Staples and Brown 2008). The potential for mtDNA introgression in *Urocitellus* to be driven by adaptive advantage of *U. parryii* mtDNA should therefore continue to be explored.

5. Conclusion

We investigated phylogeny of *Urocitellus*, a geographically widespread clade of ground squirrels whose evolutionary history has heretofore been incompletely understood. In doing so, we found the genomic history of this genus to be complex, characterized not only by pervasive ILS but also ancient hybridization among high-latitude taxa. These results represent important new insights into dynamics of *Urocitellus* diversification, but additional data are needed to fully parse these multiple drivers of gene tree discordance and thus resolve several recalcitrant phylogenetic and systematic questions. Application of genome-scale data to those issues, as well as to the issue of whether there is adaptive significance to the widespread hybridization that has been documented across Marmotini, is an important research direction to pursue.

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Figure Captions

Figure 1. Geographic distribution of the big-eared species group of *Uroditellus* in the Palearctic (left) and Nearctic (right). Different species are indicated by numbers as follows: 1) *U. undulatus*, 2) *U. parryi*, 3) *U. columbianus*, 4) *U. richardsonii*, 5) *U. beldingi*, 6) *U. armatus*, and 7) *U. elegans*.

Figure 2. Geographic distribution of the small-eared species group of *Uroditellus*. Different species are indicated by different shapes. Subspecies are indicated by numbers as follows: 1) *U. townsendii nancyae*, 2) *U. t. townsendii*, 3) *U. washingtoni*, 4) *U. brunneus brunneus*, 5) *U. b. endemicus*, 6) *U. canus canus*, 7) *U. c. vigilis*, 8) *U. mollis idahoensis*, 9) *U. m. artemesiaae*, 10) *U. m. mollis*. State abbreviations are CA=California, ID=Idaho, MT=Montana, NV=Nevada, OR=Oregon, UT=Utah, WA=Washington. Major rivers are indicated by white text.

Figure 3. Maximum clade credibility phylogram of *Uroditellus* based on Bayesian partitioned analysis of mtDNA (CYTB and control region, 1662bp total). Nodes receiving >0.95 posterior probability (PP) support indicated with closed circles. Three poorly-supported internal nodes are labeled specifically to highlight interspecies relationships. Bars at right are colored to represent recovery of species monophyly (black) or non-monophyly (gray). Sample locations (state/province) are indicated in underlined text at right of clades; all states in USA unless otherwise abbreviated (CAN=Canada, RUS=Russia, MON=Mongolia). Outgroups are *Ammospermophilus leucurus* and *Otospermophilus beecheyi*.

Figure 4. Maximum clade credibility phylogram of *Uroditellus* based on Bayesian analysis of partial von Willebrand's factor (VWF, 860bp). Nodes receiving >0.95 posterior probability (PP) support indicated with closed circles (as in Fig. 3). Bars at right are colored to represent species monophyly (black) or non-monophyly (gray). Outgroups as in Fig. 3.

Figure 5. Multidimensional scaling plot of Robinson-Foulds distances among gene tree topologies. Trees were selected randomly from gene tree posterior distributions ($N=500$ per locus; 3000 total).

Figure 6. Species tree reconstructions of *Uroditellus* using 3 different methods (*BEAST, STAR, MP-EST) based on all loci (top row) and nuclear loci only (bottom row). Trees are maximum clade credibility cladograms (i.e., w/o branch lengths). Outgroups as in Fig. 3.

Figure 7. Haplotype network based on mtDNA CYTB (1140bp; sequences compiled from this and previous studies). Taxa included are *U. parryi parryi*, *U. parryi kennicottii*, *U. elegans elegans* and *U. richardsonii*. Labels indicate the state/province of collection (AK=Alaska, CO=Colorado, MT=Montana, WY=Wyoming; AB=Alberta, MB=Manitoba, NT=Northwest Territories, NU=Nunavut, SK=Saskatchewan). Hash marks represent single mutations. Number of individuals is proportional to the size of the circle (see legend).

Figure 8. Geographic distribution of the entire *PRE* clade with major mtDNA clades indicated by color/shading. Subspecies of *U. elegans* and *U. richardsonii* are indicated with text, and subspecies of *U. parryi* are numbered as follows: 1) *U. p. ablusus*, 2) *U. p.*

kennicottii, 3) *U. p. kodiacensis*, 4) *U. p. leucostictus*, 5) *U. p. lyratus*, 6) *U. p. nebulicola*,
7) *U. p. osgoodi*, 8) *U. p. parryi*, 9) *U. p. plesius*, 10) *U. p. stejnegeri*.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1

Hypothesis	Basis	mtDNA	VWF	BRCA	FGB	GBA	GHR	*BEAST	STAR	MP-EST
big-eared monophyly	K - Nadler 1966 K - Nadler 1984	Y	N	N	N	N	N	Y/N	Y/N	Y/N
small-eared monophyly	K - Nadler 1966 mtDNA - Harrison et al. 2003	N	N	N	N	N	N	Y/Y	N/N	N/N
"townsendii complex" monophyly	M - Howell 1938 K - Nadler 1982 K - Nadler 1984	N	N	N	N	N	N	Y/N	Y/N	N/N
<i>elegans</i> monophyly	M - Robinson and Hoffmann 1975 K - Nadler 1966 K - Nadler 1971	N	Y	Y	N	Y	Y	-	-	-
<i>parryi</i> monophyly	M - Robinson and Hoffmann 1975 K - Nadler 1966 K - Nadler 1984	N	Y	Y	Y	N	Y	-	-	-
<i>mollis</i> monophyly	M - Davis 1939 K - Rickart 1985	N	N	N	N	N	N	-	-	-

Table 1. Phylogenetic hypotheses tested in this study (column 1) and their basis (column 2; M=morphology, K=karyotypic, mtDNA=mitochondrial DNA; references are those most relevant to the hypotheses presented). Columns 3-11 list the results of hypothesis tests using single gene trees (mtDNA, 5 nuclear) as well as species trees reconstructed using 3 different methods. Species tree results are in the format: all loci/nuclear loci only. The "*townsendii* complex" consists of *U. canus*, *U. mollis*, and *U. townsendii*. See text for further details.

Table 2

Species Comparison	Minimum Genetic Distance	<i>P</i>	Percentage of Subspecific Comparisons Significant
<i>U. elegans</i> – <i>U. richardsonii</i>	0.0114	0.06	1/3 (33%)
<i>U. parryii</i> – <i>U. richardsonii</i>	0.0254	0.06	2/10 (20%)
<i>U. parryii</i> – <i>U. elegans</i>	0.0254	0.09	2/30 (6%)
<i>U. mollis</i> – <i>U. beldingi</i>	0.0666	0.04	6/9 (66%)
<i>U. canus</i> – <i>U. beldingi</i>	0.0710	0.07	3/3 (100%)

Table 2. Species comparisons from posterior predictive checking in JML recovered as significant at the $P=0.1$ level. Listed are minimum empirical genetic distances found for samples within each significant species comparison and the exact significance level. The final column is a tabulation of the percentage of all possible subspecies combinations within each species comparison for which significance was found.

Holarctic ground squirrels
(Urocitellus)