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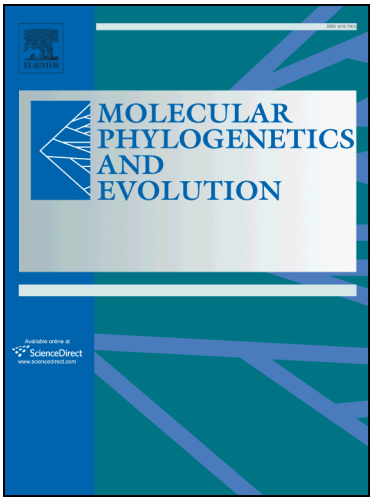

Editor's Choice Article

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Lineage Diversification of Fringe-toed Lizards (Phrynosomatidae: *Uma notata* complex) in the Colorado Desert: Delimiting Species in the Presence of Gene Flow

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Abstract

Multi-locus nuclear DNA data were used to delimit species of fringe-toed lizards of the *Uma notata* complex, which are specialized for living in wind-blown sand habitats in the deserts of southwestern North America, and to infer whether Quaternary glacial cycles or Tertiary geological events were important in shaping the historical biogeography of this group. We analyzed ten nuclear loci collected using Sanger sequencing and genome-wide sequence and single-nucleotide polymorphism (SNP) data collected using restriction-associated DNA (RAD) sequencing. A combination of species discovery methods (concatenated phylogenies, parametric and non-parametric clustering algorithms) and species validation approaches (coalescent-based species tree/isolation-with-migration models) were used to delimit species, infer phylogenetic relationships, and to estimate effective population sizes, migration rates, and speciation times. *Uma notata*, *U. inornata*, *U. cowlesi*, and an undescribed species from Mohawk Dunes, Arizona (*U.* sp.) were supported as distinct in the concatenated analyses and by clustering algorithms, and all operational taxonomic units were decisively supported as distinct species by ranking hierarchical nested speciation models with Bayes factors based on coalescent-based species tree methods. However, significant unidirectional gene flow ($2NM > 1$) from *U. cowlesi* and *U. notata* into *U. rufopunctata* was detected under the isolation-with-migration model. Therefore, we conservatively delimit four species-level lineages within this complex (*U. inornata*, *U. notata*, *U. cowlesi*, and *U.* sp.), treating *U. rufopunctata* as a hybrid population (*U. notata x cowlesi*). Both concatenated and coalescent-based estimates of speciation times support the hypotheses that speciation within the complex occurred during the late Pleistocene, and that the geological evolution of the Colorado River delta during this period was an important process shaping the observed phylogeographic patterns.

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Keywords: coalescent, conservation, gene flow, RADseq, species trees, species delimitation

1. Introduction

One of the most fundamental questions in systematic biology is also seemingly one of the simplest yet most vexing: what is a species? The significance of this question extends beyond theoretical arguments about the reality of units of nature above the organismal level. Although other measures have been growing in importance, such as phylogenetic (Faith, 1992) and genetic diversity (Moritz, 2002), the species remains the single most important unit for applied conservation efforts. Even prioritization schemes that emphasize the conservation of geographically defined high-biodiversity hotspots or wilderness areas over individual species themselves typically rely on richness or endemism of species to quantify regional biodiversity (Myers et al., 2000; Mittermeier et al., 2003). Despite this reliance on species as a measure of biodiversity, we are far from completing an inventory of the total number of species on Earth, so any technical or conceptual advances that increase the statistical rigor, objectivity and efficiency of species delimitation should have profound conservation implications (Dimmick et al., 1999; Funk et al., 2002; Adams et al., 2014).

The biological species concept (Mayr, 1942, 1963) was widely adopted by biologists during the second half of the twentieth century. However, many practicing systematists had difficulty applying the biological species concept due to its ambiguous treatment of discrete morphological or genetic variation, whereby "diagnosable populations might be ranked either as a species or subspecies, or subspecific rank itself might be applied to diagnosably distinct forms as well as to arbitrary subdivisions of clinal variation" (Cracraft, 2002, p. 131). The general lineage species concept of de Queiroz (1998, 2007, 2011), an operational extension of the evolutionary species concept (Simpson, 1951; Wiley, 1978), attempted to distinguish between the theoretical concept of species and the operational criteria that are used to put the concept into practice. While this unified concept may have engendered general agreement that species are evolutionary independent lineages, the problem remains of where to draw the line in practice. Just how much divergence, and in which traits or molecular markers, must one observe to delimit species (Hey, 2006; Hey and Pinho, 2012)? Carstens et al. (2013) advocated that systematists should explore the use of multiple of species delimitation analyses. They suggested and we agree that increased confidence should be placed in delimitation hypotheses that are supported across methods since the parameter space relevant to species delimitation exceeds the parameterization of existing methods, all of which have simplifying assumptions that may be violated in any particular system. Incongruence across different methods is evidence of either a difference in the power to detect lineages and/or violations of model assumptions in one or more analyses.

Coalescent-based approaches are now commonly used to test alternative hypotheses regarding the numbers and boundaries of species in an as statistically rigorous manner as possible (e.g. Fujita et al., 2012; Satler et al., 2013; Domingos et al., 2014; Salerno et al. 2015; Wu and Murphy, 2015; Guillemin et al., 2016; Rato et al., 2016). One promising development uses Bayes factors to rank species delimitation models with either sequence data (Grummer et al., 2014) or SNP data (Leaché et al., 2014a) in a

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coalescent-based species tree framework. A potential limitation of these methods is that they generally assume no gene flow/introgression among lineages (i.e., ancestral polymorphisms are modeled as the result of incomplete lineage sorting only), which may be frequently violated in natural systems (Hey and Pinho, 2012). Whereas Zhang et al. (2011) demonstrated that coalescent-based methods of species delimitation may be robust to low levels of gene flow, Leaché et al. (2014b) stressed that caution should be exercised in species delimitation studies since gene flow may bias species trees and parameter estimates. Thus, when gene flow may be present, additional approaches should be used that rely on thresholds based on population genetic theory. Wright (1931) calculated how migration and drift affect local allele frequencies among populations, based on the population migration rate, $2NM$, where N is the effective population size for a diploid population and M is the migration rate per gene copy per generation. For neutral markers, when $2NM < 1$, the rate of gene flow is low compared to the rate of genetic drift and divergence can proceed; conversely, when $2NM > 1$, the allele frequencies of the sink population are more likely to track those of the source (Wright, 1931; Felsenstein, 1976; Slatkin, 1985; Wang, 2004; Hey and Pinho, 2012). Using $2NM = 1$ as a cutoff for delimiting species is appealing because this statistic reflects a biologically meaningful indicator of reproductive isolation, and is independent of mutation rate and generation time, making comparisons among different organisms and genes feasible (Hey and Pinho, 2012). Indeed, this is not a new idea in systematics (e.g. Porter, 1990), but earlier workers had two major difficulties in employing this method. First, accurately calculating $2NM$ is not straightforward (Whitlock and McCauley, 1999; Hey and Pinho, 2012). Second, the calculations of Wright (1931) assume no natural selection, so using this cutoff to delimit species may be misleading if there is strong selection on some loci, as might be expected to be the case across a hybrid zone associated with a sharp ecological gradient. Fortunately, technological advances such as restriction-associated-DNA sequencing (RADseq) have increased the amount of genome-wide data available for non-model organisms by several orders of magnitude over the past few years, allowing easy access to numerous neutral markers (Pante et al., 2015), and coalescent-based isolation-with-migration models allow for the direct estimation of N and M (e.g. Gronau et al., 2011). Combined, these recent advances potentially improve the utility of $2NM$ for species delimitation studies, in conjunction with other types of analyses.

Here, we investigate the phylogenetics of fringe-toed lizards of the *Uma notata* complex (Phrynosomatidae) in the deserts of southwestern North America. *Uma* are renowned for specialized adaptations to life in desert sand dunes (Stebbins, 1944), including their namesake toe fringes that increase locomotion efficiency (Carothers, 1986). Aeolian (windblown) sand deposits occupied by *Uma* are insular, are able to migrate tens of meters downwind per year, and are tightly linked to the development of local sand sources (Blount and Lancaster, 1990; Muhs et al., 2003). As Norris (1958) recognized, these lizards represent a vexing example of the limitations that the biological species concept presents to allopatric populations. As such, the taxonomy of the *U. notata* complex has been unstable over the years, with anywhere from one to four species recognized (e.g. Norris, 1958; Trépanier and Murphy, 2001). In a pragmatic sense, this messy taxonomic history ultimately has important conservation implications. Members of *Uma*, being restricted to active dune systems, are highly susceptible to anthropogenic impacts including off-road vehicles, invasive plants such as Saharan mustard (*Brassica*

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tournefortii), obstructions to blowsand transport systems, changes in hydrology, habitat fragmentation with resulting loss of genetic diversity, and possibly climate change (Chen et al., 2006; Hedtke et al., 2007; Vandergast et al., 2015). *Uma inornata*, a flagship species for the conservation of the Coachella Valley's sand dune ecosystem (Chen et al., 2008; Alagona and Pincetl, 2008), is listed as 'threatened' under the Federal Endangered Species Act and 'endangered' under the California Endangered Species Act (USFWS, 1980, 2010). This species has lost 90-95% of its historical habitat, and is rapidly losing genetic diversity due to the coupled effects of ecological disturbance and habitat fragmentation (Vandergast et al., 2015). Other members of the *U. notata* complex face the same suite of threats to a lesser degree.

In order to introduce the hypotheses to be tested in this study, a brief review of the systematics of the *U. notata* complex is necessary. Baird (1858, p. 253) described *Uma notata* from the "Mohave Desert", which was considered to be a locality error and was localized to the "Colorado Desert" by Heifetz (1941). Baird's description of the type (USNM 4124) was deemed inadequate by Cope (1866, p. 310) who described a second specimen and its toe fringes. However, this specimen (USNM 6063) later became the type specimen of *U. scoparia* (Cope 1894, p. 435). The type locality (Fort Buchanan, Arizona) was in error – no *Uma* are known to occur there, and due to morphological characters (crescents on throat), it must have originated from the Mojave Desert, California (Heifetz, 1941). Cope (1895) described *U. inornata* from a single specimen from "the Colorado Desert, San Diego County, California", which lacks ventrolateral blotches. This locality was likely in error and the specimen almost certainly originated from the Coachella Valley of Riverside County, California (Heifetz, 1941); the type specimen (USNM 16500) was lost and later re-described (Cope, 1900). Cope (1895) described *U. rufopunctata* (cotypes USNM 21846-52, Yuma Desert, Arizona). Camp (1916, p. 516) and Van Denburgh (1922, p. 132) only recognized one species (*U. notata*). Heifetz (1941) described the subspecies *U. n. cowlesi*, identified by smaller ventrolateral blotches, from Tepoca Bay, Sonora, Mexico (CAS 53370), and united *U. rufopunctata* with *U. notata*. Norris (1958) recognized two species: *U. scoparia* and *U. notata*, and treated *U. n. inornata*, *U. notata notata*, and *U. n. rufopunctata* as subspecies. He united *U. n. cowlesi* with *U. n. rufopunctata*, although he recognized that "it is possible that it will be revived when more is known of the Sonoran coastal *Uma* from which [Heifetz] described it" (p. 281). On the basis of allozyme data that showed little variation, Adest (1977) only recognized one species, lumping all western forms (including *U. scoparia*) into *U. notata*, following Camp (1916) and Van Denburgh (1922). Utilizing morphological characters and allozymes, de Queiroz (1989, 1992) found that *U. scoparia* was sister to the *U. notata* complex (including *U. inornata*), a relationship later confirmed by sequencing mitochondrial DNA (Wilgenbusch and de Queiroz, 2000, Schulte and de Queiroz, 2008). De Queiroz (1989, 1992) recognized *U. scoparia*, *U. notata notata*, *U. n. rufopunctata*, and *U. inornata*. Trépanier and Murphy (2001), using expanded phylogeographic sampling and mitochondrial DNA (mtDNA), recognized four species within the *U. notata* complex: *U. inornata*, *U. notata* (west of the Colorado River), *U. rufopunctata* (east of Colorado River) and an undescribed species from Mohawk Dunes, Arizona that was sister to the rest of the *U. notata* complex. Stebbins (2003) recognized only two species within the *U. notata* complex: *U. inornata* and *U. notata*. Jones and Lovich (2009) recognized three species of the *U. notata* complex (*U.*

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inornata, *U. notata*, *U.* rufopunctata) and *U. scoparia*, with this taxonomy also being followed by de Queiroz and Reeder (2012) and the IUCN Red List (www.iucnredlist.org, accessed March 4, 2015).

Here, we analyzed a combination of ten nuclear loci collected using Sanger sequencing and genome-wide sequence and SNP data collected using next-generation sequencing in order to: 1) delimit the numbers and boundaries of species within the *U. notata* complex and infer a well-supported phylogeny for these species, and 2) describe how biogeographic/geological processes have been responsible for influencing speciation and demographic history. To achieve these objectives, we first analyzed these data with 'species discovery' approaches that do not require prior assumptions of species boundaries (Carstens et al., 2013), including concatenated phylogenetic and population clustering analyses, to identify potential operational taxonomic units (OTUs). We then used 'species validation' coalescent-based approaches which require assignment of individuals to different OTUs based on either species discovery methods or previously published taxonomic hypotheses. We performed Bayes factor delimitation while simultaneously estimating the species tree, then used the most strongly supported species tree as a guide tree to inform isolation-with-migration analyses to estimate divergence times, effective population sizes, and migration rates between putative species, and used these demographic values as a check for species-level status using the $2NM = 1$ threshold. Ultimately, we conservatively delimited species based on congruence across both discovery and validation methods (Carstens et al., 2013) and interpreted our results in the context of the Colorado Desert's dynamic geological and climatic history.

2. Materials and Methods

2.1. Field sampling and genomic DNA

Specimens and tissue samples were collected in the United States (California, Arizona) and Mexico (Baja California, Sonora) from 2008 to 2012. Lizards were either briefly captured to remove a piece of tail tissue before releasing the lizard otherwise unharmed, as was the case for all *U. inornata*, *U. scoparia*, and some *U. notata*, including all samples from Gottscho et al. (2014), or lizards were sacrificed as voucher specimens with MS222 injections (Conroy et al., 2009), including all Mexican samples and some *U. notata* in California. Tissues were preserved in 96-100% ethanol. All vouchers are deposited at the San Diego Natural History Museum, and tissues are stored at San Diego State University, except for *U. inornata*, which are deposited at the U.S. Geological Survey San Diego Field Station. Following the taxonomy of de Queiroz and Reeder (2012), we included 26 samples of *U. inornata*, 16 *U. notata*, 23 *U. rufopunctata* (including five samples from *U.* sp. from Mohawk Dunes and 12 representing the currently unrecognized taxon *U. cowlesi*, from the Sonoran coastal plain) and 31 *U. scoparia* as outgroups (96 total; Appendix A, Fig. 1). We used the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with RNAse A to extract genomic DNA (gDNA) following the manufacturer's instructions.

2.2. Sanger data

We included published data from ten nuclear loci, including four exons (BDNF, RAG-1, PNN, R35) and six anonymous loci (sun07, sun08, sun10, sun12, sun18, sun28)

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and amplified additional individuals for these 10 loci following Gottscho et al. (2014). Purified PCR products were sequenced on a ABI 3730XL automated sequencer (Applied Biosystems). In total, we analyzed Sanger data for 88 individuals, including 18 *U. inornata*, 16 *U. notata*, 6 *U. rufopunctata*, 12 *U. cowlesi*, five *U.* sp., and 31 *U. scoparia* (Appendix A). Sequencher v4.7 (Gene Codes Corp., Ann Arbor, MI) was used to analyze data quality, trim primer sequences, produce alignments and call heterozygous sites. PHASE v2.1 (Stephens et al., 2001) and seqPHASE (Flot, 2010) were used to determine haplotypes using default settings. DNAsp v5.10 (Librado and Rozas, 2009) was used to calculate summary statistics for the Sanger data, including number of haplotypes and nucleotide diversity (π). Because this program cannot accommodate sequences with ambiguity codes, we chose the most probable set of alleles and base calls for all sites as estimated by PHASE to estimate summary statistics.

2.3. RAD data

We collected genomic SNP data using the double-digest Restriction-Associated-DNA sequencing (ddRADseq) protocol (Peterson et al., 2012), using the enzymes and size selection window of Leaché et al. (2014a, 2015), including 13 *U. inornata*, 12 *U. notata*, six *U. rufopunctata*, 10 *U. cowlesi*, five *U.* sp., and 18 *U. scoparia* (64 total, Appendix A). DNA concentrations were measured using a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY) and assessed samples for high molecular weight by running them out on 1% agarose gels. The high-fidelity restriction enzymes SbfI and MspI (New England Biolabs, Ipswich, MA) were used to digest 200-500 ng of gDNA per lizard. Digestions were purified with Agencourt AMPure beads (Beckman Coulter, Danvers, MA) before attaching uniquely bar-coded adapters to each library with T4 Ligase (New England Biolabs, Ipswich, MA). To reduce the odds of incorrectly assigning samples due to sequencing error, each barcode differed by a minimum of two bases (assuming a Phred score of 20, the minimum quality threshold, 0.01% of reads would be incorrectly assigned). After a second AMPure purification, we generated eight pools of eight uniquely bar-coded libraries at equimolar concentrations before size selecting fragments 415-515 bp long using a Pippen Prep (Sage Science, Beverly, MA) with 2% gel cassettes. Phusion *Taq* Polymerase (New England Biolabs) was used for PCR. We used a two-step cycle (98º C for 10 seconds, 72º C for 20 seconds) 12 times, followed by a final extension step of 72º C for 10 minutes. In this way, all 64 lizards were identified using the hierarchical bar-coding scheme. An Agilent Bioanalyzer 2100 was used to ensure the libraries were at the appropriate concentration and size distribution for sequencing. We sequenced the final libraries (100 bp single end reads) on half a flow-cell lane of a HiSeq 2500 (Illumina, San Diego, CA) at the Institute of Integrative Genome Biology (University of California, Riverside).

The Python pipeline pyRAD v2.1.2 was used for RAD data quality control, alignment, and genotype calling (Eaton, 2014). The benefit of pyRAD is that it accommodates indel variation through its use of USEARCH (Edgar, 2010) and MUSCLE (Edgar, 2004), improving homolog identification across phylogenetically divergent samples (Eaton and Ree, 2013, Leaché et al., 2015). The pipeline followed seven steps: 1) raw reads were de-multiplexed by barcode; 2) restriction cut sites and adapter sequences were trimmed, bases with Phred scores less than 20 were replaced with ‘N’ and reads with more than 10 Ns were removed; 3) reads were clustered within individuals

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into putative loci with USEARCH v7.0.1090 (Edgar, 2010) using an arbitrary clustering threshold of 0.85, retaining only loci with at least 10x coverage and with 10 or fewer Ns; 4) error rate and heterozygosity were jointly estimated; 5) consensus base calling was performed using the mean error and heterozygosity rates of step four, while consensus sequences with more than five heterozygous sites were excluded (first paralog filter); 6) consensus sequences were clustered across individuals using MUSCLE v3.8.31 (Edgar, 2004) with a 0.85 clustering threshold; 7) loci in which more than 50% of samples had shared heterozygosity at a site were removed (second paralog filter), and alignments of both full loci (89 bp) and variable sites (individual SNPs) were generated in a variety of formats (Nexus, Phylip, Structure, etc.) for further analysis.

There is still no consensus how to treat missing data in analyses of RADseq data – excessive levels of missing data may lead to spurious results from principal components analysis (PCA) and other clustering algorithms (Rheindt et al., 2014), wherein individuals with high levels of missing data may erroneously group out as separate populations, but low tolerance for missing data may bias the mutational rate spectrum of sampled loci, so that slowly evolving loci are disproportionately represented in the final dataset (Huang and Knowles, 2014). Also, concatenated phylogenetic analyses have been shown to benefit from longer alignments even with a higher percentage of missing data (Wagner et al., 2013). Given this uncertainty, we generated two datasets with differing levels of completeness: a larger, less complete matrix with 1,614 loci represented by at least 40 of 64 individuals (hereafter “relaxed”), and a smaller matrix retaining 597 loci (581 biallelic) represented by at least 60 of 64 individuals (hereafter “stringent”).

2.4. Concatenated phylogenetic inference

We inferred phylogenies from concatenated RADseq data using two different methods in order to identify putative lineages to be later validated using species delimitation models. First, a ML phylogeny of the concatenated data was generated with RAxML v8.1.1 (Stamatakis, 2014). This rapid approach does not require *a priori* assignment of individuals to populations or species (de Queiroz and Gatesy, 2007) and thus is well suited to inform subsequent species tree analyses. However, combining unlinked loci into a supermatrix and forcing a bifurcating hierarchy at the population level may result in erroneous groupings, so shallow-scale relationships and branch lengths were interpreted cautiously. Using the relaxed dataset, we concatenated 1,614 loci that resulted in a 149,784 bp alignment with 7,752 SNPs and 15.86% missing data. We implemented the GTR+Γ substitution model (unpartitioned), performed 500 rapid bootstrap replicates, and rooted the tree with *U. scoparia* as the outgroup.

To estimate a time-calibrated phylogeny, we used BEAST v2.1.2 (Bouckaert et al., 2014) to perform an unpartitioned Bayesian analysis of the concatenated relaxed dataset with a uniform Yule model tree prior and the GTR+Γ substitution model with empirical base frequencies. A strict clock was rejected using the coefficient of variation for the clock rate, so a lognormal relaxed clock was used. Calibrating the molecular clock for RADseq data is problematic because all loci are anonymous and no useful fossil calibrations are currently available within *Uma* or close relatives (de Queiroz, 1989). Thus, we estimated a genome-wide neutral mutation rate (μ) using a fossil-calibrated phylogeny of iguanian lizards (Townsend et al., 2011) based on 29 nuclear exons for 47 iguanian and 29 outgroup taxa, including *Uma* and other phrynosomatids. We extracted

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the mean estimate of the rate for the third codon positions extracted from the log file from Townsend et al. (2011) under the assumption that the third position is typically under the least amount of natural selection and thus is probably closest to the background mutation rate, although it probably is an underestimate of the true neutral rate. Averaging across 29 loci, we obtained a mean estimate of 7.57 x 10^{-9} substitutions/site/year, which was used for all subsequent dating analyses. The Markov chain was run for 50 million generations, sampling trees every 1,000 steps. Convergence of two independent runs was assessed using Tracer v1.6 to ensure that effective sample size (ESS) values for the posterior and likelihood were >200 (Rambaut et al., 2014). After discarding 30% of the trees as burn-in from each run, a maximum clade consensus tree was generated from the combined data using LogCombiner v1.8.1 and TreeAnnotator v1.8.1 (Bouckaert et al., 2014).

2.5. Clustering analyses

In order to guide subsequent species delimitation analyses, we used both parametric and non-parametric approaches to detect genetic structure without any *a priori* hypotheses about individual assignment. To infer structure with the Sanger data, we utilized the R package Geneland v4.0.3 (Guillot, 2008; Guillot et al., 2005, 2008). Individual genotypes were encoded using the numbered alleles output by PHASE v2.1 (entire sequences were coded as numerical alleles, rather than individual SNPs within sequences). We used the correlated allele frequencies model (Falush et al., 2003; Marchini et al., 2004; Guillot et al., 2008) within Geneland, and performed five independent runs with K_{max} values ranging from 2 to 6 (attempts to increase K beyond this did not converge). The Markov Chain Monte Carlo (MCMC) was run for 20 million generations, sampling every 1,000 generations, discarding the first 10% as burn-in.

For the RADseq data, first we conducted a PCA using smartPCA v1.02.10 (Patterson et al., 2006; Reich et al., 2008), analyzing 581 unlinked biallelic SNPs (one per locus) represented in the stringent dataset. The main benefit of PCA is its ability to detect genetic structure without the computational burden of Bayesian clustering algorithms and the absence of assumptions about the underlying population genetic model, but it does not provide a group assessment. Therefore, we also used Discriminant Analysis of Principal Components (DAPC) implemented in the R package adegenet v2.0-0 to assign individuals to populations using the stringent dataset (Jombart et al., 2010). DAPC uses PCA as a prior step to Discriminant Analysis (DA) to transform the data so that variables submitted to DA are uncorrelated and fewer than the number of sampled individuals, allowing DA to be applied to genome-wide SNP data. This approach follows a two-step process. First, we conducted the PCA, retained the first 64 PCs, and followed the recommendations of Jombart et al. (2010) in using the Bayesian Information Criterion (BIC) to select the optimal number of clusters from K=2 to K=10. Second, K-means clustering was implemented by retaining the first 20 PCs retaining >80% of the cumulative variance and the first four discriminant functions to assign individuals to clusters.

Admixture v1.23 (Alexander et al., 2009) was used to detect population structure among identified clusters from the stringent dataset. This maximum likelihood-based program implements the same underlying population genetic model as Structure (Pritchard et al., 2000). While both programs assign individuals into clusters in an attempt to minimize linkage disequilibrium and maximize Hardy-Weinberg equilibrium,

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Admixture has the added benefit of a fast numerical optimization algorithm to decrease computational time while avoiding problems with MCMC convergence. We used the cross-validation procedure to select the optimal K value (Alexander et al., 2009), testing values ranging from 2 to 10.

2.6. Species delimitation with Bayes factors

We tested eight different species delimitation models (Fig. 2) ranging from two species (*U. scoparia* and *U. notata*) to six species (*U. scoparia*, *U. inornata*, *U. notata*, *U. rufopunctata*, *U. cowlesi*, and *U.* sp.). Intermediate models B-G lumped the five species of the *U. notata* complex in various combinations, including all previously published taxonomies summarized in the introduction, as well as novel groupings suggested by species discovery approaches (i.e., concatenation-based phylogenies and clustering algorithms). We first used Bayes factor delimitation (BFD, Grummer et al., 2014) to test alternative species delimitation models with the phased Sanger data. For each model, the species tree was first estimated with *BEAST (Heled and Drummond, 2010) implemented in BEAST v1.8.1 (Drummond et al., 2012), using an unpartitioned HKY+G substitution model with empirical base frequencies for all loci, a strict molecular clock (the coefficient of variation under a relaxed clock could not reject the strict clock), a Yule process species tree prior, a MCMC chain length of 300,000 generations, sampling the tree space every 10,000 generations, and assessing convergence with Tracer v1.6. Marginal likelihoods for each model were estimated using both the stepping-stone and path sampling methods, with 100 path sampling steps of 100,000 generations each, logging parameters every 1,000 generations (Baele et al., 2012). For the RADseq data, we used BFD* (Leaché et al., 2014a) to test the eight species delimitation models with the program SNAPP v1.2.5 (Bryant et al., 2012) implemented in BEAST 2.3.1, which infers a species tree using biallelic SNP data while bypassing gene tree inference in a fully coalescent-based model. However, it does not accommodate missing data at the species level, so a custom R script was used to generate a dataset of 468 presumably unlinked biallelic SNPs present in a minimum of one individual for each of the six putative species listed above. We used a gamma distribution for mutation rate priors and lognormal distributions for the lambda priors, and upper bounds on parameters were left to their default values. We conducted path sampling with 10 steps and a MCMC chain length of 100,000 steps (20,000 pre-burn-in) to estimate the marginal likelihood (averaged across two independent runs. For the BFD and BFD* analyses, we assessed the strength of support of alternative species delimitation models following the scale of Kass and Raftery (1995). A positive BF statistic (2ln) favors model 1, whereas a negative BF value favors model 2. The scale is as follows: $0<2\ln(BF)<2$ is negligible support, $2<2\ln(BF)<6$ is weak but positive support, $6<2\ln(BF)<10$ is strong support, and $2\ln(BF)>10$ is decisive support.

2.7. Species tree inference of Uma

In order to inform the guide tree for isolation-with-migration models (section 2.8), our species tree analysis of *Uma* was based on the results of the Bayes factor-based species delimitation analyses described above, using the best supported set of *U. notata* complex species (including the outgroup, *U. scoparia*). We constructed a coalescent-based species tree using the Sanger data (ten nuclear loci) with *BEAST (Heled and

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Drummond, 2010) implemented in BEAST v1.8.1 (Drummond et al., 2012). We initially ran the analysis with a lognormal relaxed clock, but because the 95% HPD for the coefficient of variation for the substitution rate parameter included zero for each locus, we could not reject a strict molecular clock, so for the final analysis a strict clock was assumed for all loci. For the coalescent-based species tree using RADseq data, we used SNAPP v.1.1.5 in BEAST v2.1.2, with a gamma rate distribution on the SNAPP prior. The upper bounds on other parameters were set at default values. The MCMC was run for 500,000 generations, sampling every 1,000 generations. For both analyses, we conducted two independent runs and assessed for convergence with Tracer v1.6, and we produced maximum clade credibility trees of the combined posterior distribution with LogCombiner v1.8.1 and TreeAnnotator v1.8.1 after discarding the first 10% of trees as burn-in from each run. The posterior distribution of trees from each dataset was visualized with DensiTree v2.2 (Bouckaert, 2010).

2.8. Isolation-with-migration models

We estimated population demographic parameters, including migration rates (m), divergence times (τ), and effective population sizes (θ) using G-PhoCS (Generalized Phylogenetic Coalescent Sampler) v1.2.3 (Gronau et al., 2011). Initially, we attempted to include all species/individuals in a single model, but these parameter-rich analyses failed to converge on a stable posterior distribution. Thus, we split the dataset to simplify the models. For each separate model, we repeated the last step of pyRAD v2.1.2 to create a complete dataset (i.e. no missing data) for each model with a subset of individuals representing geographically adjacent OTUs (Fig. 3). Model A (605 loci, 25 individuals) included *U. inornata* and *U. notata* with two migration parameters. Model B (540 loci, 28 individuals) included *U. notata*, *U. rufopunctata*, and *U. cowlesi*, with two migration parameters between *U. notata* and *U. rufopunctata* and two between *U. rufopunctata* and *U. cowlesi*. Model C (606 loci, 21 individuals) included *U. rufopunctata*, *U. cowlesi*, and *U.* sp., with two migration parameters between *U.* sp. and *U. rufopunctata* and two between *U.* sp. and *U. cowlesi*. Originally Model C also included *U. notata*, and attempts to run models with two additional migration parameters between *U. rufopunctata* and *U. notata* (six in total) failed to converge, but as these parameters were estimated in Model B this is not a major concern. The prior probability distributions for all models assumed a gamma distribution. For τ and θ we used α=1, β=10000 (except for ancestral θ, where we set β=20000) and for m we used α=0.002, β=0.00001. All fine-tune parameters were set automatically. For each model, we ran two replicate analyses of 1 million MCMC steps each, discarding the first 10% as burnin and combining the posterior distributions across runs. Tracer v.1.6 was used to ensure that all ESS values were >200 and that the combined runs converged on the same posterior distributions for all parameters.

Following Gronau et al. (2011), we used the equation $T = \tau/\mu$ (where $\mu = 7.57 \times 10^{-9}$ substitutions/site/year as described above) to convert τ into divergence time in years; to estimate effective population sizes in numbers of diploid individuals (N) we used $\theta = 4N\mu g$, where g is the average generation time, in this case, two years (Mayhew 1966); we used $M = m\mu g$ to convert raw migration rates into per-generation migration rate. As a test to validate species, we calculated mean, 95% low, and 95% high HPD for the population migration rate ($2NM$) and interpreted the results as follows: if mean $2NM > 1$, we rejected the hypothesis that the OTU constitutes a distinct species; likewise, if mean $2NM < 1$, we

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confirmed the hypothesis that the OTU represents a separate species. Results of this test were interpreted to have strong statistical support if the 95% HPD intervals did not span 1, otherwise the mean values of 2*NM* were interpreted cautiously.

3. Results

3.1. Raw data characteristics

The combined length of all Sanger loci was 5,214 bp (mean 521.4 bp) containing 172 segregating sites (mean of 17.2/locus) and 220 total haplotypes (mean 22/locus) (Table 1). *Uma cowlesi*, *U. rufopunctata*, and *U. notata* had comparably high mean nucleotide diversity (0.47%, 0.46% and 0.45%, respectively) while *U. scoparia* and *U.* sp. had the lowest (0.09% and 0.12%, respectively). *Uma inornata*, despite its small range and endangered status, had intermediate diversity (0.26%). For the RADseq data, we obtained a final alignment with 1,614 loci present in a minimum of 40 of 64 individuals, resulting in a 149,784 base-pair (bp) matrix containing 7,752 SNPs, and a second alignment with 616 loci and 2,805 SNPs represented by at least 60 of 64 individuals, which we further pruned to 581 unlinked biallelic SNPs. As with the Sanger data, *U. cowlesi*, *U. rufopunctata*, and *U. notata* had a comparably high percentage of heterozygous sites (0.41%, 0.43%, and 0.35%, respectively), while *U. scoparia* had the lowest (0.15%), and *U.* sp. (0.22%) and *U. inornata* (0.25%) were intermediate (Appendix B).

3.2. Concatenated phylogenetic analyses

The ML estimate of the phylogeny based on RADseq data shows that five of the six geographically distinct OTUs shown in Figure 1 are supported as monophyletic (exclusive) with >70% bootstrap support (Fig. 4). The sole exception is *U. rufopunctata*, which is paraphyletic (non-exclusive), with the individuals forming a graded series leading to a *U. notata+U. inornata* clade. Bootstrap support values within these six OTUs are mostly low, but strongly supported clades were revealed within *U. scoparia* (northwestern Mojave Desert [1-16]) and within *U. notata* (Laguna Salada in Baja California [42-43]). The ML results are mostly corroborated by the Bayesian time tree analyses (Fig. 5), which also found strong support for the exclusivity of the same five OTUs (>0.95 posterior probability). This topology only differs from the ML tree at the shallowest (weakest supported) nodes within OTUs. The *U. notata* complex was estimated to have diverged from *U. scoparia* in the late Pleistocene (0.54 – 1.01 Ma), well after the formation of the modern Salton Trough and Colorado River delta. Initial divergence within the *U. notata* complex was estimated to occur entirely during the late Pleistocene (0.54 – 0.84 Ma; Fig. 5).

3.3. Genetic clustering

Geneland analysis of the Sanger data under K_{max}=5 clustered *U. scoparia* (not shown), *U. inornata*, *U. notata* + *U. rufopunctata*, *U. cowlesi*, and *U.* sp. (Fig. 6). Results for other values of K_{max} are not shown as figures, but are described here as follows: under K_{max}=4, *U. cowlesi* clustered with *U. notata* + *U. rufopunctata*, while under K_{max}=3, *U. inornata* was lumped in as well. Only under K_{max}=2 was *U.* sp. finally assigned to the rest of the *U. notata* complex. Under K_{max}=6, the program was no longer able to assign individuals to populations with high confidence (PP > 90).

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The Admixture analysis of 581 unlinked SNPs supported K=6 as the optimal model (CVE=0.264; Table 2; Fig. 5). Under K=6, *U. scoparia* was split into two clusters that correspond with the northwestern and southeastern populations in the concatenated phylogenies, and *U. inornata*, *U. notata*, *U. cowlesi*, and *U.* sp., were also recognized as discrete clusters. All individuals of *U. notata* and *U. cowlesi* were assigned to their respective OTUs with high confidence, while *U. rufopunctata* was shown to be composed of admixed individuals from *U. notata* and *U. cowlesi*.

In the smartPCA analysis based on 581 unlinked SNPs, the first principal component axis (PC1) separates *U. scoparia* from the *U. notata* complex, while PC2 corresponds with a northwest/southeast axis, clearly separating *U. inornata* and *U.* sp. as distinct groups, but separation between *U. cowlesi*, *U. rufopunctata* and *U. notata* are not as clearly distinguishable in this plot (Fig. 7). However, PC3 and PC4 were better able to distinguish these groups, especially *U. cowlesi*. The PC space between *U. rufopunctata* and *U. notata* is blurred by two individuals (32,49) with similar PC4 scores. Overall, in PC space, the genetic affinities of *U. rufopunctata* and *U. notata* is suggestive of Model G (Fig. 2). The DAPC supported K=5 under the BIC (Fig. 5, Table 2). The results of this analysis are consistent with the results of Trépanier and Murphy (2001), corresponding with Model F in Figure 2 where *U. cowlesi* and *U. rufopunctata* are combined into a single population.

3.4. Species delimitation with Bayes factors

Bayes factor species delimitation analyses (BFD and BFD*) decisively supported the most species rich Model H (6 sp.) over the next best model (BF > 10), and in all cases, the simplest model A (2 sp.) was ranked the lowest (Table 3). The methods differed in their relative rankings of the intermediate models, although in all cases models with more species were ranked higher. The results of this test, assuming no or limited gene flow, demonstrate that the *Uma* sampled in this study are best modeled as six evolutionary distinct lineages or species, including *U. scoparia* and five species within the *U. notata* complex.

3.5. Species tree analyses

Both the *BEAST analyses of Sanger data and the SNAPP analysis of SNP data found strong support for the monophyly of the *U. notata* complex with respect to *U. scoparia*, corroborating the concatenated analyses presented here (Figs 4, 5) and previously published hypotheses (Fig. 8). However, the *BEAST analyses of Sanger data had much weaker power to resolve interspecific relationships (Fig. 8), which is perhaps unsurprising given the much smaller size of this dataset. Yet none of the strongly supported nodes disagree with those supported in the SNAPP analysis of RAD data, which estimated relationships with a high degree of confidence. The topology of the species tree based on SNP data was largely consistent with the concatenated trees, except that the species tree has no way of demonstrating the non-exclusivity of *U. rufopunctata* with respect to *U. notata* and *U. inornata*. The biogeographic pattern of the SNAPP tree mirrors the concatenated trees – the OTUs form a pectinate series with the southern and eastern populations diverging earliest and the northwestern ones latest.

3.6. Isolation-with-migration models

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The effective population sizes (*N*), population migration rates (2*NM*), and divergence times (*T*) estimated with the three isolation-with-migration models are shown in Table 4. First, all population divergence times were more recent than the concatenated BEAST estimates, and lie in the very late Pleistocene, with mean estimates ranging from 55.8 Ka for the divergence of *U. rufopunctata* from *U. cowlesi* to 78.8 Ka for the divergence of *U. notata* from *U. inornata*. Mean estimates for *N* ranged from 3,400 individuals for the ancestor of *U. rufopunctata* and *U. cowlesi* to 115,312 for extant *U. cowlesi*. For Model A, migration was shown to be asymmetrical between *U. notata* and *U. inornata*; although some gene flow was estimated from *U. inornata* to *U. notata*, the entire 95% HPD for $2NM < 1$. In Model B, again migration was asymmetrical, as both *U. notata* and *U. cowlesi* showed high gene flow into *U. rufopunctata* ($2NM > 1$), although only the estimate from *U. notata* was strongly supported. Also, whereas there appears to be high gene flow into *U. rufopunctata* from *U. cowlesi* and *U. notata*, there appears to be no gene flow out of *U. rufopunctata* in either direction ($2NM < 0.01$). Finally, Model C showed that $2NM < 1$ in both directions between *U.* sp. and both of its neighbors, *U. cowlesi* and *U. rufopunctata*, and this result was robustly supported by the 95% HPD intervals.

4. Discussion

4.1. Species delimitation

Carstens et al. (2013) argued that systematists should use a wide range of species delimitation analyses and place their trust in delimitation hypotheses that are supported across methods. Following this logic, Table 5 summarizes the various OTUs tested for species level status, and whether or not the results of the analyses support recognizing that OTU as a species or not. This comparison makes apparent that *U. inornata* and *U.* sp. (as well as the outgroup, *U. scoparia*) all are unanimously supported for species level status by all nine analyses. Thus, we recommend the continued recognition of *U. inornata* as a separate species, and we support the hypothesis of Trépanier and Murphy (2001) that *U.* sp. constitutes a cryptic species – a formal written description of type specimens, although outside the scope of this manuscript, is urgently needed and forthcoming. We note that previous studies have shown that both *U. inornata* and *U.* sp. are diagnosable (Norris, 1958; Trépanier and Murphy, 2001), the former by morphology and mtDNA, the latter presumably just by mtDNA (13 fixed differences). The results conflict concerning the recognition of *U. rufopunctata*, and its relationship to *U. cowlesi* and *U. notata*. Although models separating these three taxa performed the best in BFD and BFD*, the results of the clustering (species discovery) analyses consistently fail to identify *U. rufopunctata* as a distinct cluster: 1) Admixture supports it as a hybrid zone between *U. cowlesi* and *U. notata*, 2) Geneland groups it with *U. notata* and the PCA analysis also suggests a greater genetic similarity between *U. rufopunctata* and *U. notata*, and 3) DAPC groups it with *U. cowlesi*. The results of the isolation-with-migration model show significant gene flow ($2NM > 1$) from both *U. notata* and *U. cowlesi* into *U. rufopunctata*. In fact, there appears to be greater gene flow into *U. rufopunctata* from *U. notata* than from *U. cowlesi*. This could explain the suggested greater genetic similarity between *U. notata* and *U. rufopunctata* in the Geneland and PCA analyses, as well as the seemingly greater phylogenetic affinity of the *U. rufopunctata* individuals to *U. notata* in

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the concatenated RADseq phylogenetic analyses (Fig. 4, 5) and the species tree analyses (Fig. 8). Although our analyses support *U. rufopunctata* as being a hybrid zone between *U. notata* and *U. cowlesi*, our analyses also support *U. cowlesi* and *U. notata* as distinct independent evolutionary lineages; both lineages are exclusive in the concatenated phylogenetic analyses and there is no gene flow between *U. cowlesi* and *U. notata* [via *U. rufopunctata*], as shown in the Admixture and G-PhoCS analyses. Given these results, we advocate the recognition of *U. cowlesi* and *U. notata* as distinct species, but *U. rufopunctata* is not a distinct species, but instead appears to represent a hybrid zone/population (="*U. notata* x *cowlesi*"), perhaps similar to the secondary contact zone between western whiptail lizards (*Aspidoscelis tigris* and *A. marmoratus*) in the Cochise filter barrier of southwestern New Mexico (Dessauer et al., 2000; de Queiroz and Reeder, 2012), although in this case, Pleistocene migration of the Colorado River delta rather than post-glacial range expansion is likely the underlying process driving hybridization (see section 4.3). Further study to determine the biological nature of this hybrid zone is warranted.

4.2. Phylogeny

Both ML and Bayesian analyses of concatenated RAD data resolved the following features in common with each other: 1) *U. scoparia* is monophyletic and sister to the *U. notata* complex, which is also strongly supported as monophyletic, 2) individuals from Mohawk Dunes (*U.* sp.) are supported as monophyletic and are sister to the remainder of the complex, 3) individuals from the Gran Desierto/Yuma Dunes (*U. notata* x *cowlesi*) are paraphyletic with respect to the *U. notata*+*U. inornata* clade, both of which occur west of the Colorado River, and which are each monophyletic sister lineages. These results are largely consistent with the published mtDNA tree of Trépanier and Murphy (2001), except that the mtDNA data were unable to resolve "*U. rufopunctata*" from *U. cowlesi*. The topology of the species trees inferred using different data and methods of analysis (ten nuclear loci and *BEAST vs. 468 unlinked biallelic SNPS and SNAPP) were partially congruent with each other (Fig. 8). The topology of the species tree inferred using SNAPP was strongly supported and consistent with the mtDNA and concatenated trees, showing a hierarchal nested pattern wherein the southeastern species diverged earliest, starting with *U.* sp., while the northwestern species (*U. inornata*) originated latest. By contrast, the species tree inferred using ten nuclear loci with *BEAST did not support this hierarchal relationship (Fig. 8), as it shows *U. cowlesi* as sister to *U.* sp., but this arrangement was weakly supported (PP = 0.72). Thus we favor the SNAPP tree because this topology is based on the much larger SNP dataset that provides strong support (PP = 1.0) at all nodes. These results demonstrate the power of next-generation sequencing approaches to obtain enough data to resolve phylogenetic relationships among closely related species with high support.

4.3. Historical Biogeography

An unsolved issue in the historical biogeography of the American southwest revolves around how complex Miocene and Pliocene geological events, including the evolution of the San Andreas Fault system, the Baja California Peninsula, the Gulf of California, and the delta of the Colorado River, influenced speciation of desert flora and fauna (Mulcahy et al., 2006; Wood et al., 2013; Gottscho et al., 2014). These large-scale

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landscape changes may have driven speciation directly by vicariance, such as the Tertiary flooding of the Salton Trough as it expanded along the San Andreas Fault system (Elders et al., 1972; McDougall et al., 1999). Alternatively, limits to dispersal across a landscape previously shaped by large-scale events, especially Pleistocene glacial cycles, may have been the major driver of speciation and differentiation in the southwestern aridlands (Wood et al., 2013). Based on nuclear sequence data, Gottscho et al. (2014) estimated the divergence date between *U. scoparia* and the *U. notata* complex to be in the mid-Pleistocene (0.99 Ma; 95% HPD: 0.60 – 1.34 Ma), which broadly overlaps with the concatenated analyses presented here (0.76 Ma; 95% HPD: 0.54 – 1.01 Ma). The topology and timing of the chronogram (Fig. 5) strongly supports the hypotheses that *U. scoparia* in the northern part of their range and *U. inornata* in the Coachella Valley were both the results of northwestern range expansions in the late Pleistocene (Norris, 1958). Within *U. scoparia*, individuals from Pinto Basin (Joshua Tree National Park) and Dale Lake (Fig. 4, individuals 1-3) are nested deep within the Mojave River clade, sister to an individual from Barstow along the Mojave River (individual 4), indicating that these populations likely descended from individuals that dispersed back southeast from the Mojave River, following a sand transport corridor through Twenty-nine Palms. The timeline for this expansion largely matches that of the divergence and expansion of *U. inornata* in the Coachella Valley (Fig. 5). These dating estimates, combined with the late Pleistocene divergence times within the *U. notata* complex estimated from isolation-with-migration models (Table 4), allow us to reject the hypothesis that earlier Pliocene/Miocene geological events, such as marine inundations of the Salton Trough (Buising, 1990; McDougall et al., 1999), were responsible for diversification of *Uma* in the Colorado Desert.

Since *Uma* are restricted to windblown sand habitats, the processes governing the deposition and movement of dunes during the Pleistocene likely influenced population structure and speciation. During glacial maxima, ample precipitation produced perennial lakes and streams, most notably the Colorado River, that eroded sediment from the mountains. The climate during these periods, although more mesic than present (Thompson and Anderson, 2000), still allowed for desert communities to endure, although the extent of these communities is controversial (Cole, 1986; Holmgren et al., 2014). During dry, warm interglacial periods, sediments were exposed to the wind, allowing for the accumulation and migration of dune systems (Blount and Lancaster, 1990; Muhs et al., 2003). An additional complication is that the Colorado River itself was not stable during this time. Throughout the earlier part of the Pleistocene, the Colorado River delta was positioned further east of its eastern location, discharging into the Gulf of California in the vicinity of modern Bahia Adair (Fig. 1). In the late Pleistocene, as the Salton Trough continued to expand and deepen due to rifting along the San Andreas Fault system, the delta shifted westward to its current location west of the Gran Desierto (Blount and Lancaster, 1990), and occasionally drained as far northwest as the prehistoric Lake Cahuilla in the center of the Salton Trough, currently occupied by the Salton Sea (Waters, 1983). The instability of the Colorado River throughout climatic fluctuations of the Pleistocene is consistent with our findings of asymmetrical gene flow across this barrier, resulting in a broad hybrid zone between *U. notata* and *U. cowlesi*.

4.4. Conservation implications

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The results of this study support recognizing *U. inornata* as a distinct species, and to recognize as a new species the populations from Mohawk Dunes. The mean effective population sizes of the federally threatened *U. inornata* and *U.* sp. from Mohawk Dunes (8,894 and 9,452) are much lower than those estimated for the other extant lineages in our study. This may be of conservation concern, as *U.* sp. is almost entirely restricted to the Barry M. Goldwater Air Force Range in the Sonoran Desert of Arizona, although the northernmost portion of its range extends into BLM lands near Interstate 8 (where the samples in this study were collected). The small range of this species and its location on a bombing and gunnery range make it vulnerable to military training activity. However, the U.S. Air Force already has extensive experience dealing with other sensitive species such as pronghorn antelope on this range (Krausman et al., 2005), so continued responsible range management is essential to ensure that this species remains merely vulnerable and not threatened or endangered with extinction. We note that *U.* sp. is similar to *U. inornata* in terms of geographic range and effective population size, and is even more isolated phylogenetically. To recognize and conserve the geographic population structure evident within the *U. notata* complex, we recommend evaluating the three geographic units for status as management units or distinct population segments: 1) *Uma notata* northwest of the modern Colorado River (*Uma notata*), 2) a central group in the Gran Desierto between the Colorado River and the El Pinacate Crater (*U. notata x cowlesi* = "*U. rufopunctata*"), and 3) a group southeast of El Pinacate and Bahia Adair (*U. cowlesi*) (Fig. 1), recognizing that these geographically isolated populations probably are currently exchanging genes or have done so in the recent past.

Levels of genetic diversity are of prime importance for conservation efforts, as low diversity may result in reduction in fitness and inability to adapt to a changing environment (e.g. Dobrynin et al., 2015). Both nucleotide diversity of our Sanger data (Table 1) and heterozygosity of our RADseq data (section 3.1) revealed that *U. inornata* and *U.* sp. have the lowest genetic diversity within the *U. notata* complex (albeit not as low as *U. scoparia*). Continued monitoring of genetic diversity within these species may help to determine whether measures to conserve diversity may become necessary in the future (Vandergast et al., 2015).

5. Conclusions

We utilized recent advances in next generation sequencing and coalescent-based species delimitation to study fringe-toed lizards of the *Uma notata* species complex, a group of special conservation concern with a long and unstable taxonomic history. We argue for the recognition of *U. inornata*, *U. notata*, *U. cowlesi*, and an undescribed species from Mohawk Dunes, Arizona (*U.* sp.), but we recommend treating *U. rufopunctata* as a hybrid population (*U. notata* x *cowlesi*). Divergence dating estimates using both concatenated and coalescent-based approaches indicate that speciation within this complex took place during the late Pleistocene, a time when glacial cycles and shifts in the Colorado River conspired to generate many of the dune formations observed today. This study highlights the continued importance of basic systematic biology for applied biodiversity conservation efforts, and the need for caution in interpreting incongruent results in species delimitation studies.

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Data Accessibility

Upon acceptance of this manuscript, all raw sequence data and intermediate files necessary to replicate our analyses will be deposited in the Dryad Digital Repository (http://www.datadryad.org).

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Table 1. Summary statistics for phased Sanger data. L = length of locus, N = number of sequences, S = number of segregating (polymorphic) sites, h= number of unique haplotypes, and π = nucleotide diversity for each OTU.

Locus	**L**	**N**	**S**	**h**	$\pi_{scoparia}$	$\pi_{inornata}$	π_{notata}	$\pi_{rufopunctata}$	$\pi_{cowlesi}$	$\pi_{sp.}$
BDNF	613	166	8	10	0.0000	0.0020	0.0015	0.0019	0.0016	0.0000
PNN	600	130	18	20	0.0012	0.0026	0.0038	0.0042	0.0032	0.0000
R35	450	154	18	26	0.0019	0.0022	0.0063	0.0065	0.0076	0.0000
RAG-1	660	152	15	14	0.0010	0.0003	0.0010	0.0007	0.0010	0.0008
sun07	530	142	16	19	0.0001	0.0006	0.0012	0.0028	0.0032	0.0004
sun08	555	84	12	13	0.0008	0.0014	0.0036	0.0025	0.0040	0.0000
sun10	599	130	32	37	0.0012	0.0034	0.0066	0.0120	0.0090	0.0039
sun12	359	152	17	24	0.0005	0.0002	0.0063	0.0052	0.0023	0.0014
sun18	379	74	12	14	0.0006	0.0095	0.0085	0.0018	0.0069	0.0000
sun28	469	152	24	43	0.0018	0.0039	0.0058	0.0090	0.0085	0.0056
MEAN	521	134	17.2	22	0.0009	0.0026	0.0045	0.0046	0.0047	0.0012
TOTAL	5,214	1,336	172	220	0.0092	0.0259	0.0445	0.0465	0.0474	0.0121

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Table 2. Bayesian information criterion (BIC) and Cross-validation error (CVE) values for different K values estimated from Discriminant Analysis of Principal Components (DAPC) and Admixture, respectively.

K	BIC	CVE
2	222.16	0.303
3	217.29	0.274
4	212.81	0.272
5	**209.86**	0.272
6	210.50	**0.264**
7	212.61	0.285
8	213.93	0.303
9	216.28	0.314
10	217.54	0.334

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Table 3. Results of the Bayes Factor Delimitation (BFD) analyses using Sanger data (Grummer et al., 2014) with path sampling (PS) and stepping-stone (SS) of marginal likelihoods, and BFD* analyses using RAD data (Leaché et al., 2014a) using PS. Bayes factor (BF) values are used to rank species models relative to the species model with the lowest marginal likelihood (ML). Model H (bold) was decisively supported as the best-fit model (BF = 0) under all three methods.

	BFD (PS)			**BFD (SS)**			**BFD* (PS)**		
Model	**ML**	**Rank**	**BF**	**ML**	**Rank**	**BF**	**ML**	**Rank**	**BF**
A	-10252.1	8	560	-10277.0	8	564	-8890.9	8	3501
B	-10105.5	6	267	-10129.7	6	270	-8071.3	6	1862
C	-10180.3	7	417	-10204.6	7	419	-8170.7	7	2061
D	-10019.8	4	96	-10043.8	4	98	-7745.4	5	1210
E	-10034.6	5	125	-10058.6	5	127	-7438.5	4	596
F	-9980.5	2	17	-10003.7	2	18	-7211.8	2	143
G	-9988.4	3	33	-10011.7	3	34	-7214.8	3	149
H	**-9971.9**	**1**	**0**	**-9994.9**	**1**	**0**	**-7137.9**	**1**	**0**

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Table 4. Results of G-PhoCS models. *N* = effective population size (number of individuals), *T* = divergence time (years), 2*NM* = effective population migration rates, HPD = Highest Posterior Density.

Model A

Parameter	Mean	95% HPD low	95% HPD high
$N_{U.\ inornata}$	8,894	6,859	11,012
$N_{U.\ notata}$	76,271	66,890	85,839
$N_{U.\ inornata+U.\ notata}$	51,641	44,265	59,034
$T_{U.\ inornata/U.\ notata}$	78,877	53,410	107,180
$2NM_{U.\ inornata \rightarrow U.\ notata}$	0.03	0	0.16
$2NM_{U.\ notata \rightarrow U.\ inornata}$	0.60	0.34	0.95

Model B

Parameter	Mean	95% HPD low	95% HPD high
$N_{U.\ notata}$	69,356	61,004	77,951
$N_{U.\ rufopunctata}$	67,787	53,876	81,963
$N_{U.\ cowlesi}$	115,312	101,999	128,817
$N_{U.\ notata+U.\ rufopunctata}$	3,534	171	8,467
$N_{U.\ notata+U.\ rufopunctata+U.\ cowlesi}$	75,188	68,122	82,385
$T_{U.\ notata/U.\ rufopunctata}$	69,062	61,524	76,694
$T_{(U.\ notata+U.\ rufopunctata)/U.\ cowlesi}$	69,234	61,779	76,818
$2NM_{U.\ rufopunctata \rightarrow U.\ cowlesi}$	0	0	0.01
$2NM_{U.\ cowlesi \rightarrow U.\ rufopunctata}$	1.56	0.74	2.63
$2NM_{U.\ rufopunctata \rightarrow U.\ notata}$	0	0	0.01
$2NM_{U.\ notata \rightarrow U.\ rufopunctata}$	3.54	2.22	5.12

Model C

Parameter	Mean	95% HPD low	95% HPD high
$N_{U.\ rufopunctata}$	109,320	93,945	125,269
$N_{U.\ cowlesi}$	113,482	99,404	127,666
$N_{U.\ \mathrm{sp.}}$	9,452	7,824	11,092
$N_{U.\ rufopunctata+U.\ cowlesi}$	3,400	112	8,170
$N_{U.\ rufopunctata+U.\ cowlesi+U.\ \mathrm{sp.}}$	86,445	79,152	93,899
$T_{U.\ rufopunctata/U.\ cowlesi}$	55,773	48,841	62,753
$T_{(U.\ rufopunctata+U.\ cowlesi)/U.\ \mathrm{sp.}}$	55,799	48,865	62,791
$2NM_{U.\ rufopunctata \rightarrow U.\ \mathrm{sp.}}$	0.01	0	0.04
$2NM_{U.\ \mathrm{sp.} \rightarrow U.\ rufopunctata}$	0.12	0	0.36
$2NM_{U.\ cowlesi \rightarrow U.\ \mathrm{sp.}}$	0.09	0	0.57
$2NM_{U.\ \mathrm{sp.} \rightarrow U.\ cowlesi}$	0.01	0	0.04

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Table 5. Summary of results of species discovery and validation approaches to species delimitation; 'yes' indicates that the taxon (columns) was supported by the analysis (rows), while 'no' indicates that it was not.

	U. inornata	***U. notata***	***U. rufopunctata***	***U. cowlesi***	***U.* sp.**	***U. scoparia***
Discovery Approaches						
RAxML	yes	yes	no[1]	yes	yes	yes
BEAST	yes	yes	no[1]	yes	yes	yes
Geneland	yes	no[2]	no[2]	yes	yes	yes
Admixture	yes	yes	no[3]	yes	yes	yes
PCA	yes	no[2]	no[2]	yes	yes	yes
DAPC	yes	yes	no[2]	no[2]	yes	yes
Validation Approaches						
BFD	yes	yes	yes	yes	yes	n/a
BFD*	yes	yes	yes	yes	yes	n/a
$2NM < 1$	yes	yes	no	yes	yes	n/a

[1]Non-exclusive lineage leading to a *U. inornata*+*U. notata* clade.
[2]These two taxa were suggested as a single population.
[3]This taxon was suggested to be an admixed population between *U. cowlesi* and *U. notata*.

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Figure Captions

Fig. 1. Approximate historical geographic distributions of the six operational taxonomic units (OTUs) within the genus *Uma* examined in this study. Four of the OTUs (*U. scoparia*, *U. inornata*, *U. notata*, and *U. rufopunctata*) are currently recognized as species (de Queiroz and Reeder, 2012), while the other two are not. **Uma cowlesi* (Heifetz 1941; Tepoca Bay, Sonora, Mexico) was described as a subspecies of *U. notata* with an associated type series but later united with *U. n. rufopunctata* by Norris (1958), yet our analyses suggest that this taxon may be valid, so it is treated as a species. **The proposed species from the Mohawk Dunes, Arizona (Trépanier and Murphy, 2001) has yet to be formally named and is here designated *U.* sp.

Fig. 2. Eight alternative species delimitation hypotheses tested for *Uma* in this study. Rows represent the six operational taxonomic units shown in Figure 1, while columns represent the species delimitation models A–H. Model A represents the simplest model, treating the *U. notata* complex as a single species sister to *U. scoparia* (the outgroup), while the most species rich model (H) treats each OTU as a separate species. All other tested models are based on previously published taxonomies or results of the Geneland analyses under different values for the maximum number of populations (K_{max}) as follows: Model A (2 sp., Norris, 1958), Model B (3 sp., Stebbins, 2003), Model C (3 sp., Geneland, K_{max}=3), Model D (4 sp., Jones and Lovich, 2009), Model E (4 sp., Geneland, K_{max}=4), Model F (5 sp., Trépanier and Murphy, 2001), Model G (5 sp., Geneland, K_{max}=5), and Model H (6 sp.).

Fig. 3. Three alternative isolation-with-migration models tested using G-PhoCS v1.2.3 (Gronau et al., 2011). τ = divergence time scaled by mutation rate, θ = effective population size scaled by mutation rate, generation time, and effective population size, and m = migration scaled by mutation rate and generation time.

Fig. 4. Left: Maximum likelihood (ML) tree estimated with RAxML v8.1.1 using the "relaxed" concatenated RADseq dataset (1,614 concatenated loci). Right: Map of the 64 individuals with RAD sequence data. Colored names at tips correspond to individual field numbers (Table 1), and the numbers in brackets correspond to the numbered points on the map. Numbers above nodes represent bootstrap support values based on 500 rapid replicates under the GTR+GAMMA model.

Fig. 5. Bayesian chronogram of using the "relaxed" concatenated RADseq dataset (1,614 concatenated loci) aligned with the results of Discriminant Analysis of Principal Components (DAPC) under K=5 and Admixture under K=6 using the stringent dataset. The shaded bars on nodes represent 95% Highest Posterior Density (HPD) confidence intervals.

Fig. 6. Results of the Geneland analysis of *Uma notata* complex populations under K_{max}=5 showing maps of the posterior probability (PP) of population assignment (the fifth cluster, *U. scoparia*, is not shown). All individuals were assigned to their respective OTUs with PP > 0.9.

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Fig. 7. Principal Components Analysis (PCA) plots of the "stringent" RADseq dataset (581 unlinked SNPs). PC1, PC2, PC3, and PC4 represent 21.5%, 7.1%, 5.4%, and 4.8% of the variance in the dataset, respectively.

Fig. 8. *Uma* species trees for the six OTUs under the most species rich delimitation model (H). On the left are the maximum clade credibility trees, and on the right are the posterior distributions after burn-in visualized with DensiTree (blue represents the dominant topology while green and red represent alternative topologies). **A)** species tree estimated using *BEAST and 10 nuclear loci, and **B)** species tree estimated with SNAPP and 468 unlinked SNPs .

Appendix Captions

Appendix A. Basic data for *Uma* specimens included in this study. GPS coordinates are not provided for *U. inornata*, which is a federally threatened species, but interested parties may contact the authors for this information for legitimate purposes (AUR = available upon request).

Appendix B. Summary statistics for RAD data. N_{reads} = total number of reads after demultiplexing, P_{total} = total number of reads that passed quality filter, C_{total} = total number of clusters (0.85 similarity threshold), D_{total} = average depth of the total clusters, C_{pass} = number of clusters that passed the coverage threshold (10x), D_{pass} = average depth of coverage of clusters that passed the threshold, F_{loci} = number of loci with >10x depth and passed paralog filter, N_{sites} = number of sites across all loci, N_{het} = number of heterozygous sites, Het = percentage of sites that are heterozygous.

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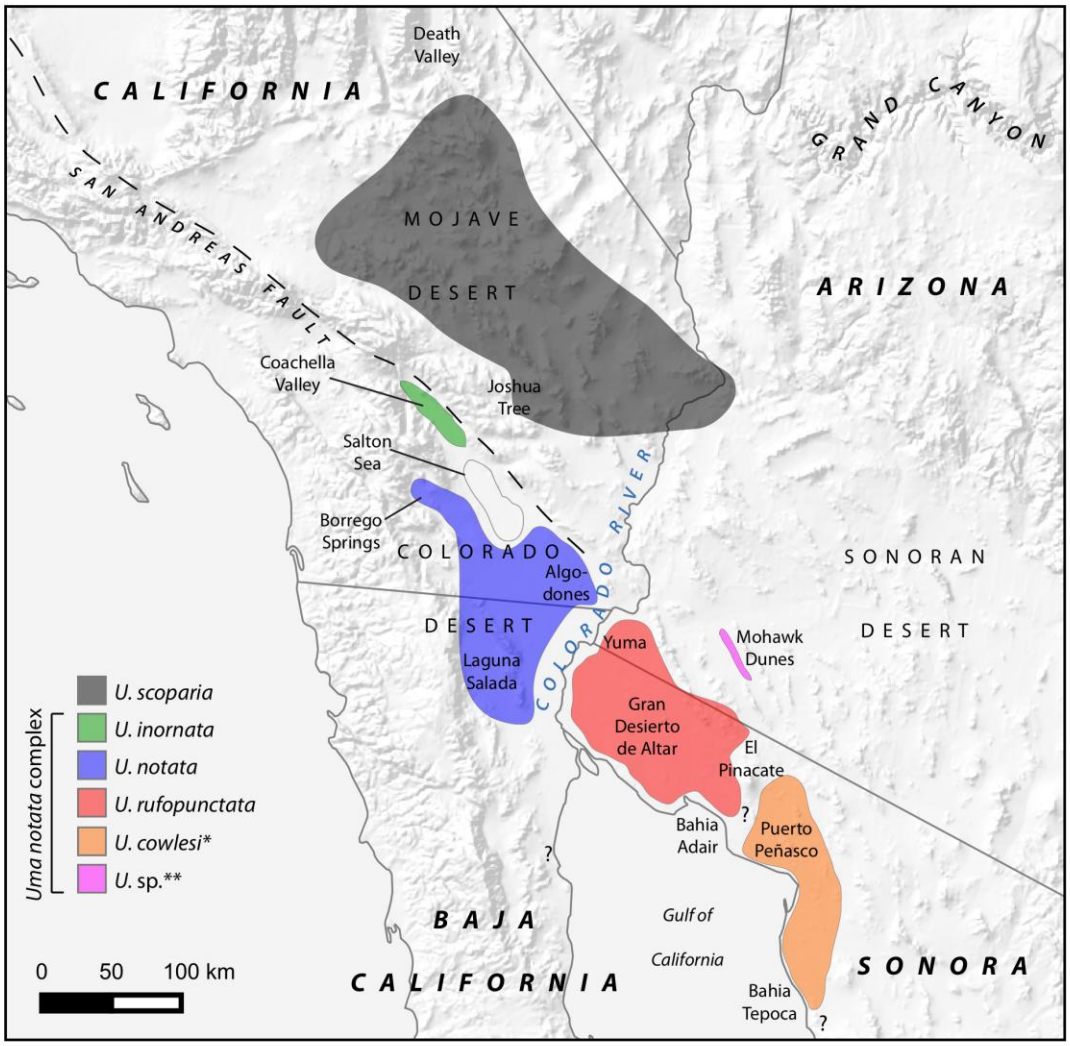

Fig. 1.

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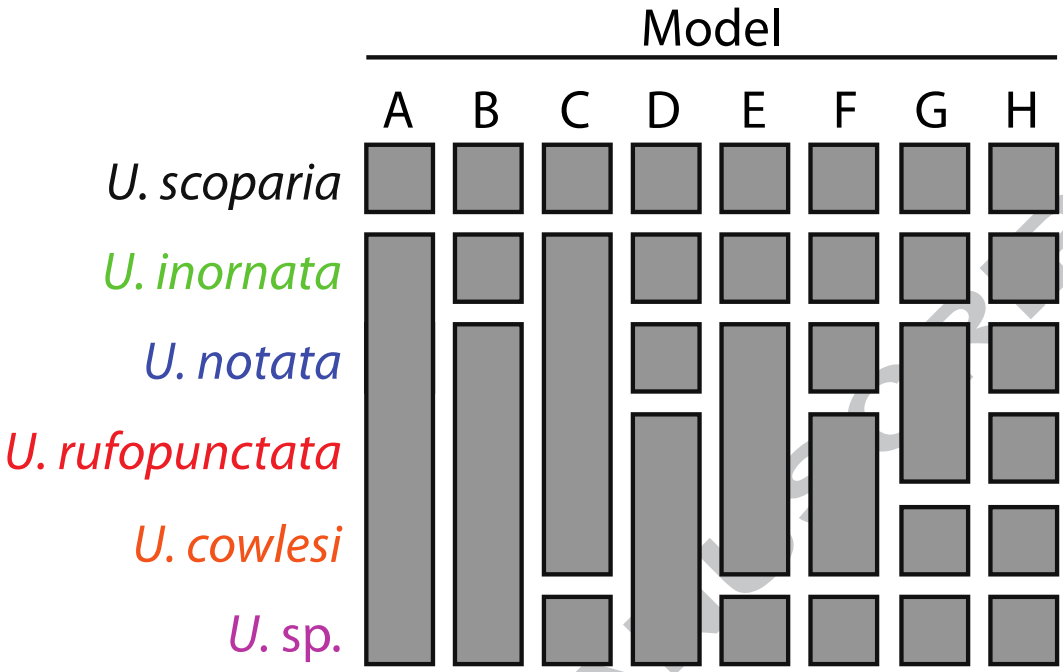

Fig. 2.

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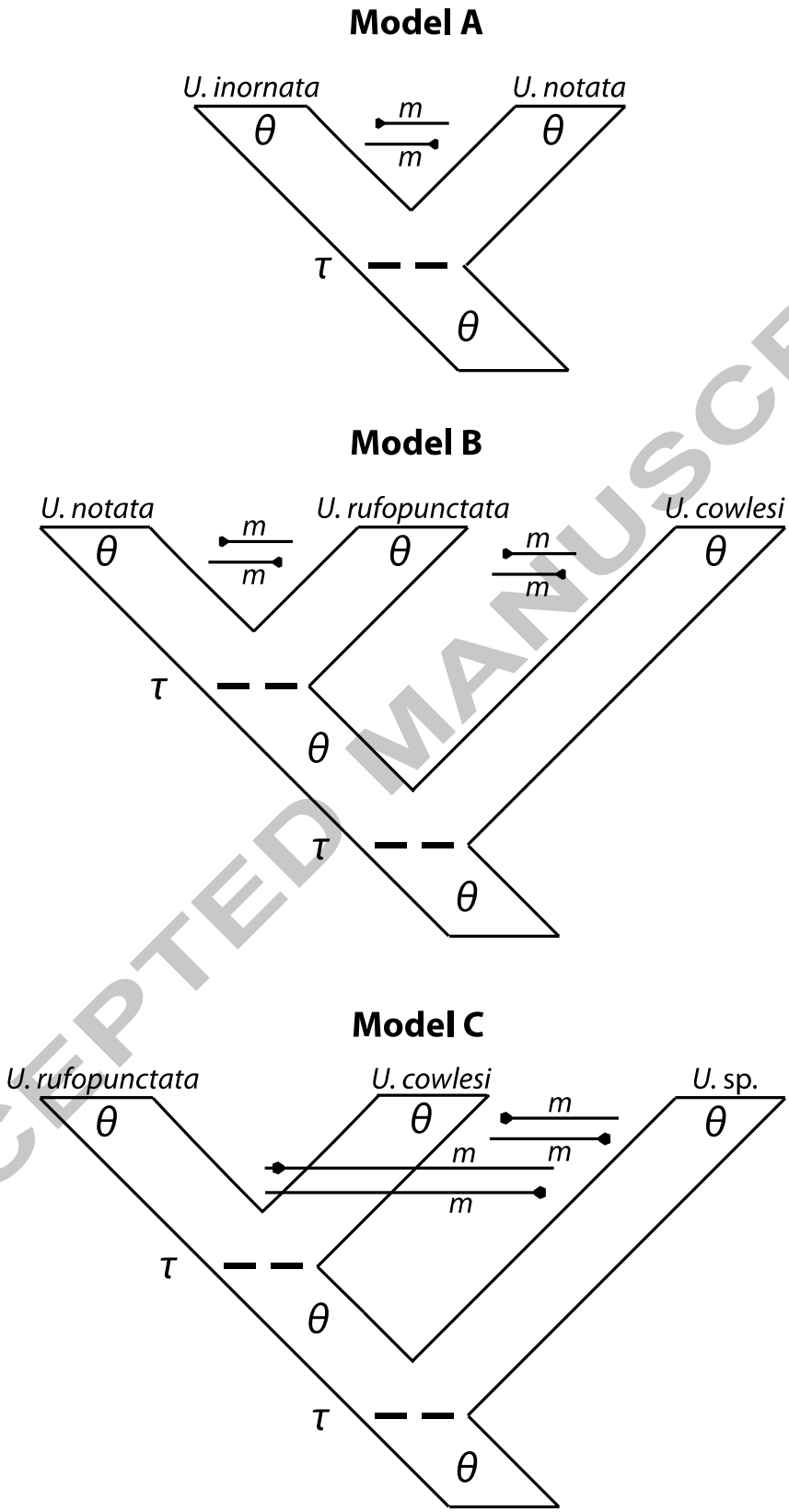

Fig. 3.

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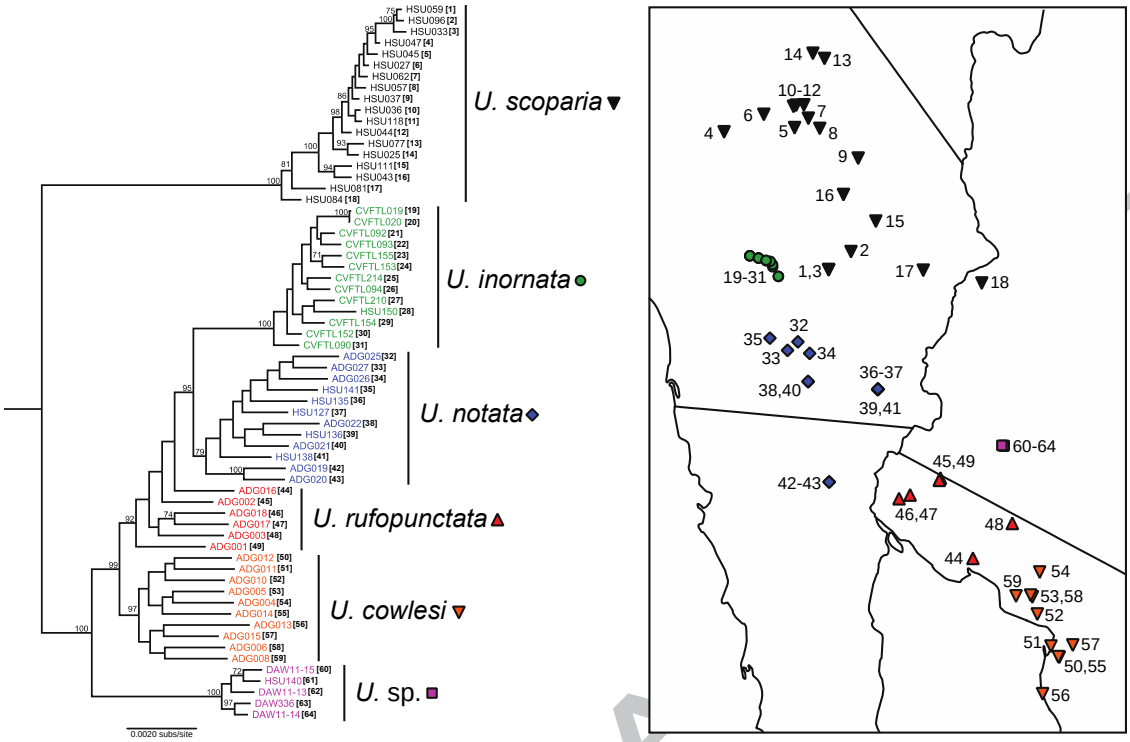

Fig. 4.

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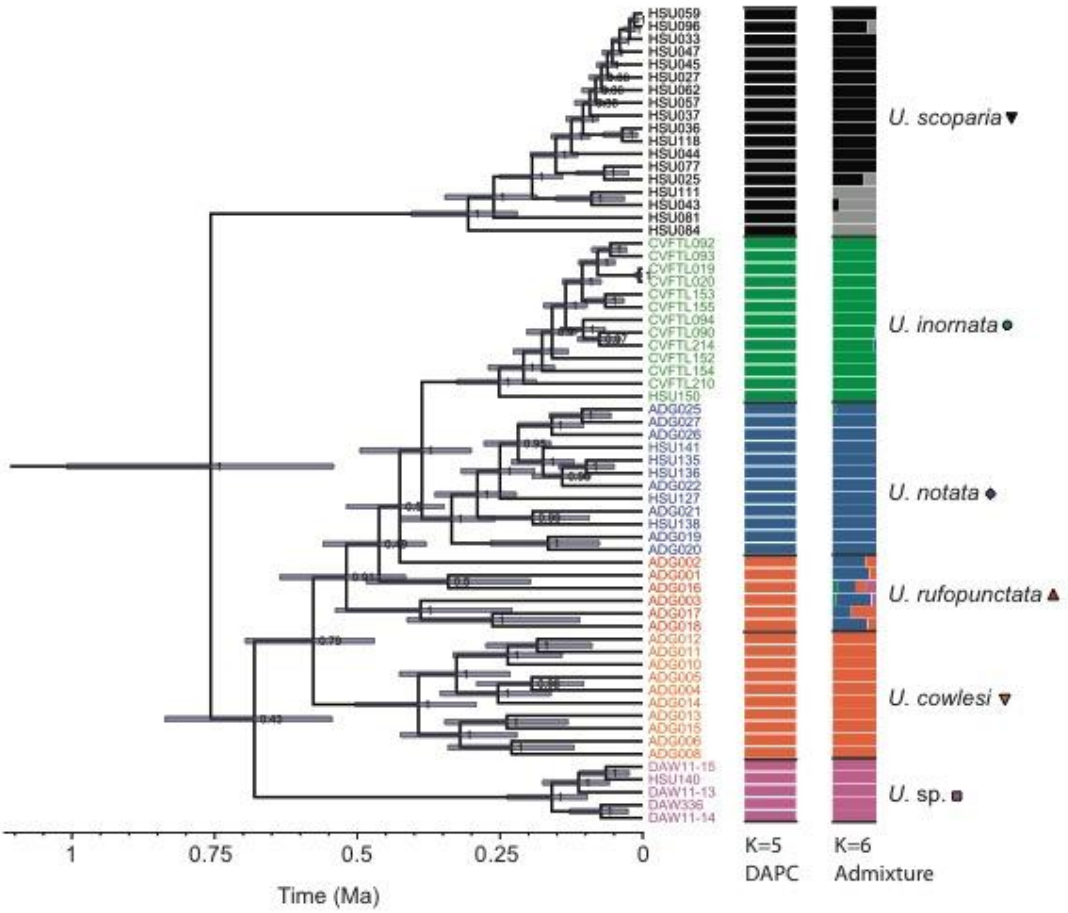

Fig. 5.

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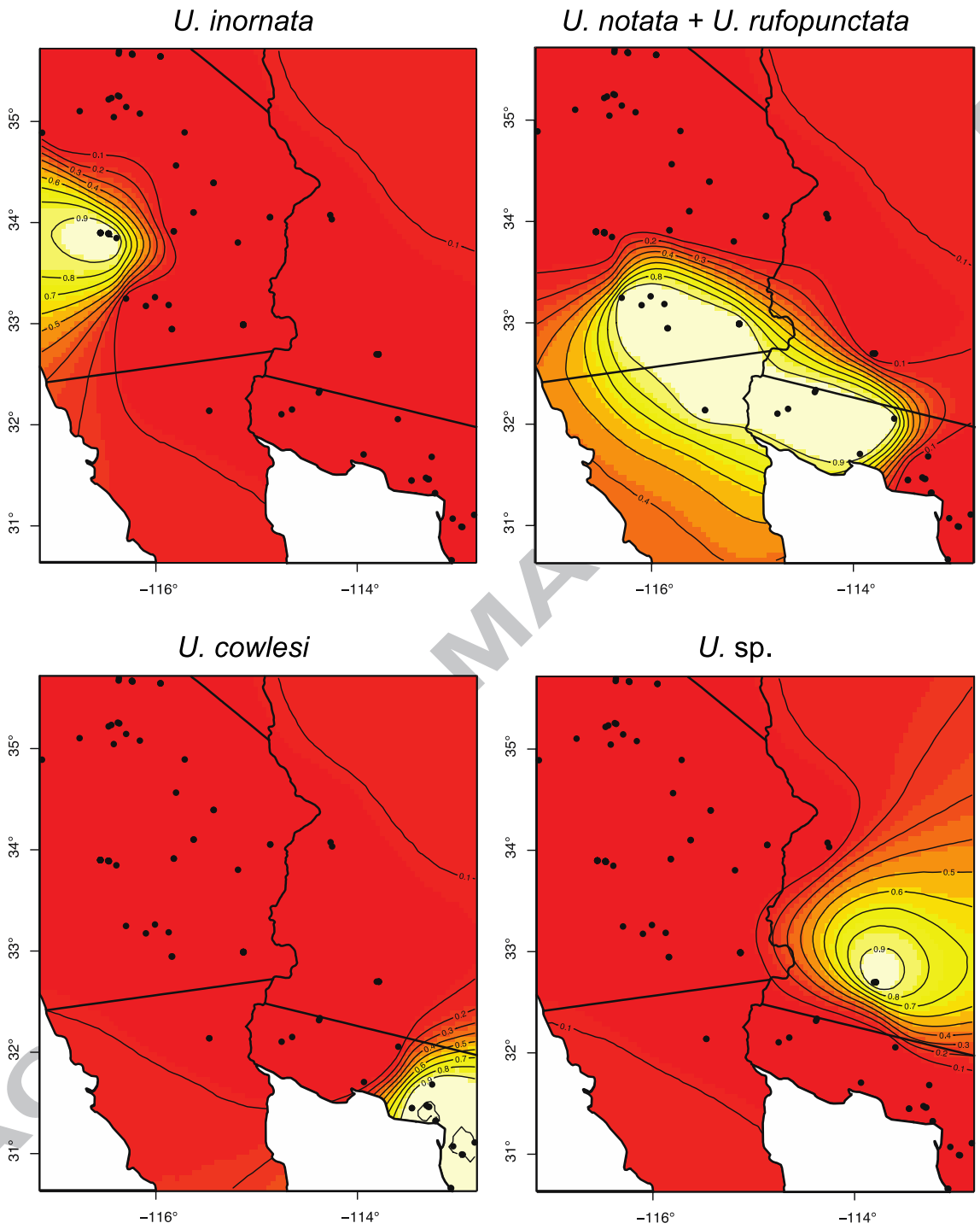

Fig. 6.

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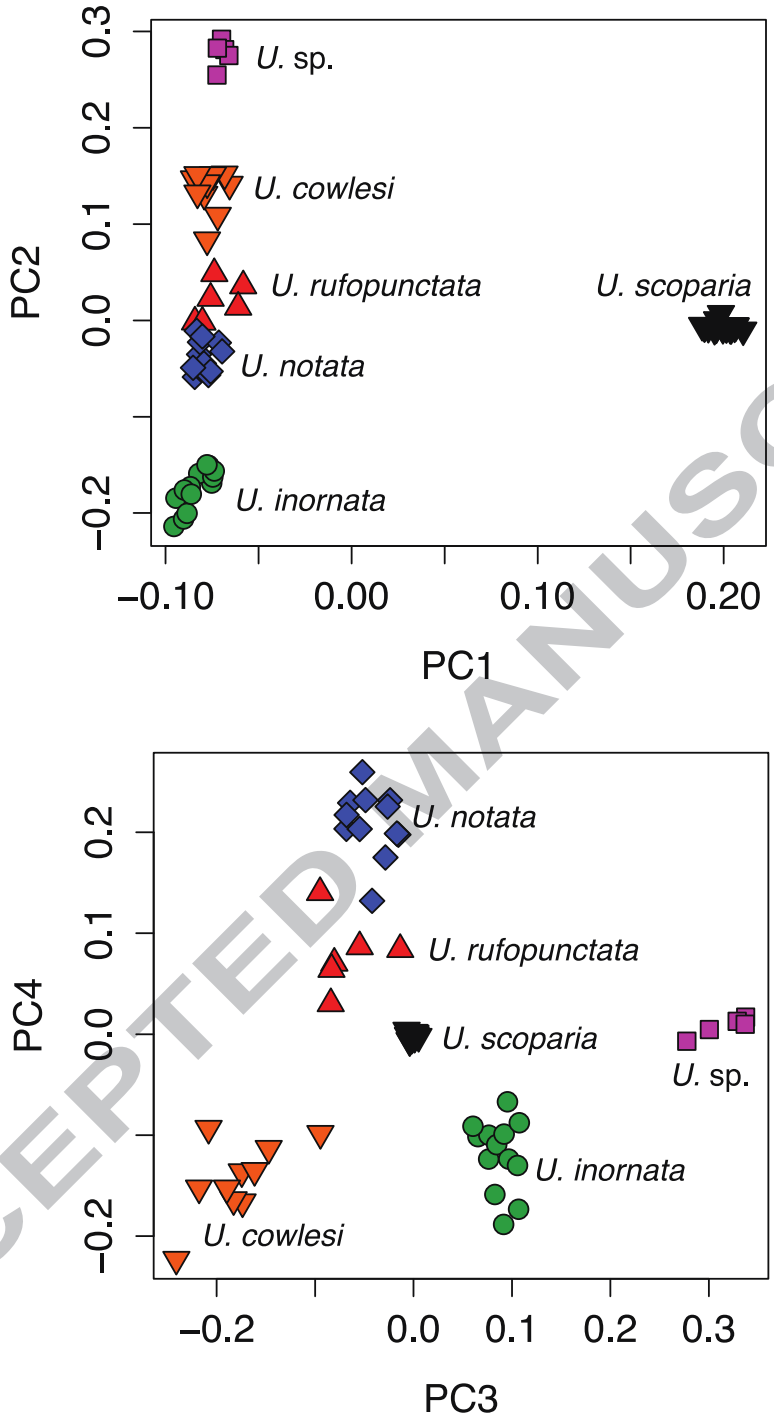

Fig. 7.

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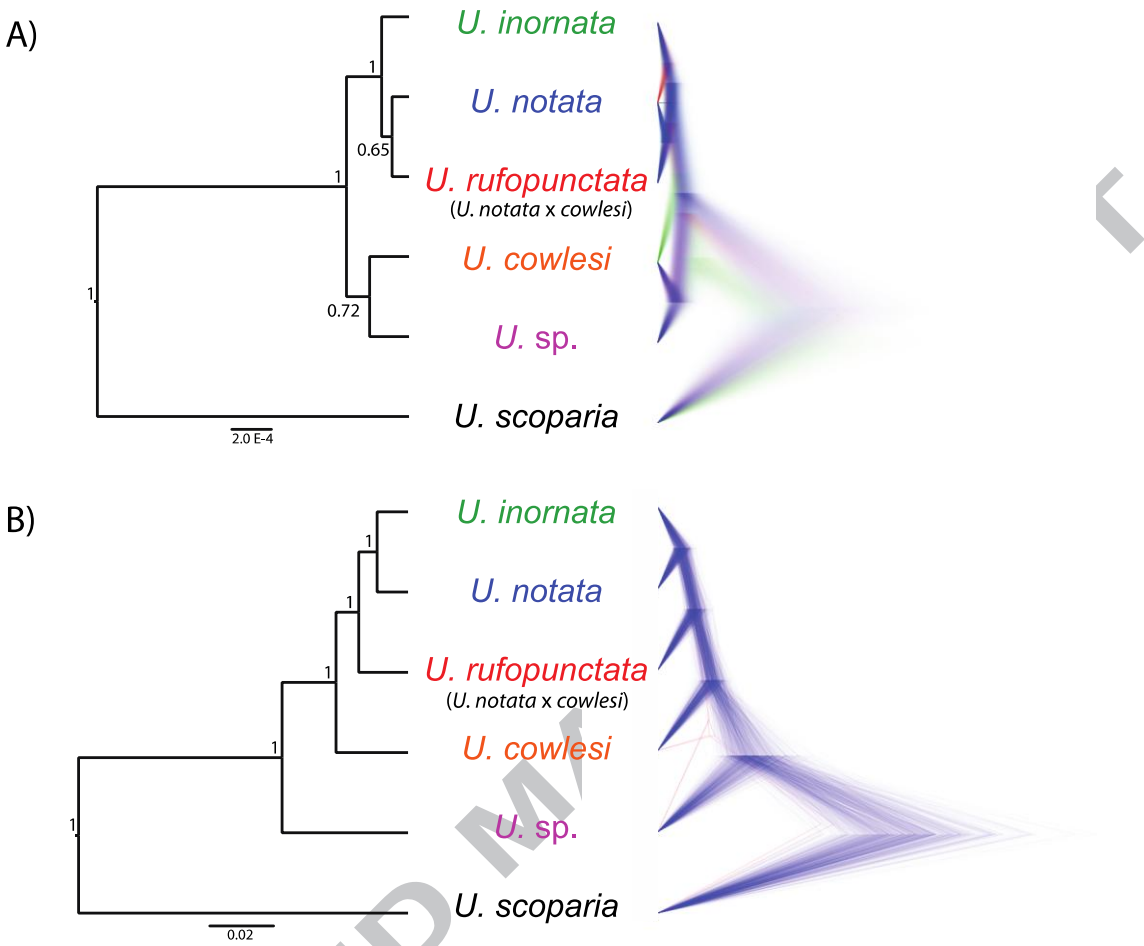

Fig. 8.

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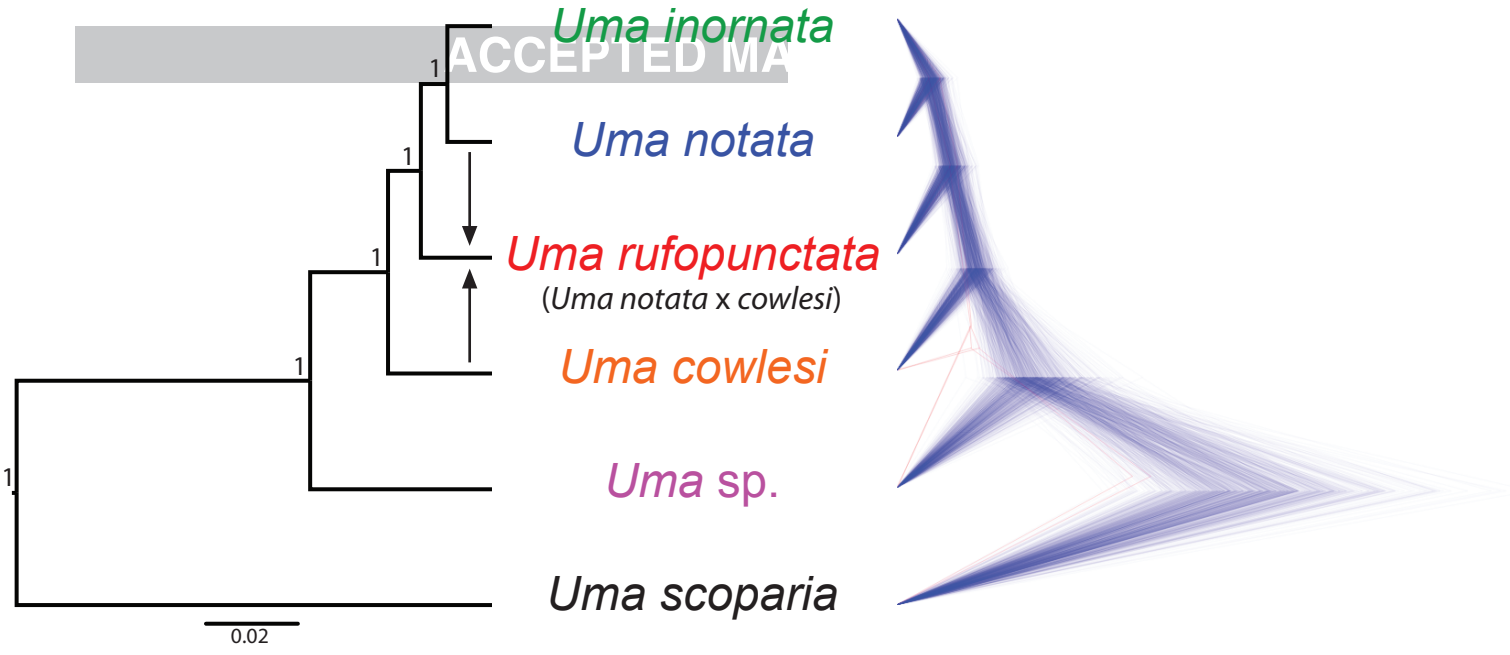
Uma inornata
Uma notata
Uma rufopunctata
(Uma notata x cowlesi)
Uma cowlesi
Uma sp.
Uma scoparia
1
1
1
1
1
0.02

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Highlights

- Four species are delimited within the *Uma notata* complex.
- There is an undescribed species (*U.* sp.) from Mohawk Dunes, Arizona.
- The federally threatened species *U. inornata* is supported as distinct.
- *Uma rufopunctata* is treated as a hybrid population (*U. notata* x *cowlesi*).
- Pleistocene timing of speciation is supported within the complex.

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