JOURNAL OF NATURAL HISTORY, 2018
https://doi.org/10.1080/00222933.2018.1429689

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Integrative taxonomy at the nexus of population divergence and speciation in insular speckled rattlesnakes

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ABSTRACT

We describe two diminutive species of rattlesnakes (genus *Crotalus*) from small nearshore islands off the coast of Baja California in the western Gulf of California, Mexico. In order to test the hypothesis that some island populations represent cohesive species entities, we applied linear discriminant analysis and uniform validation procedures to multiple classes of intrinsic trait data. By using previously recognised species to establish a threshold for species recognition, we found that assignment of specimens to either new species was as probable as with other established rattlesnake species within the speckled rattlesnake (*Crotalus mitchellii*) complex. We also found that assignment of specimens from other island populations was not as probable as for the established species, and these populations are referable to *C. pyrrhus*. The species endemic to Piojo Island is most closely related to other island and mainland populations of *C. pyrrhus* whereas the species endemic to Cabeza de Caballo Island is apparently most closely related to *C. angelensis*, a nearby island endemic of large body size. However, patterns from both mitochondrial and nuclear phylogenies, and phenotypic variation, indicate that evolutionary trajectories of both of these species have been influenced by introgression from *C. angelensis*. We speculate that collective evidence based on contrasting patterns of nuclear and mitochondrial evolution supports a hybrid origin of the species from Cabeza de Caballo Island followed by exceptionally rapid mitochondrial evolution. Consistent with small body size, both species show a reduction in various scale counts relative to other species of the *C. mitchellii* species complex, suggesting that dwarfism is not simply a plastic response to insular conditions.

http://www.zoobank.org/urn.lsid:zoobank.org:pub:FBC8A11B-04A3-4231-85CA-3972DF5A42FF

ARTICLE HISTORY
Received 6 November 2017
Accepted 22 December 2017
Online 16 February 2018

KEYWORDS
Island speciation; Viperidae; Squamata; *Crotalus polisi* sp. nov; *Crotalus thalassoporus* sp. nov

Introduction

Inferring speciation in insular isolates is rarely straightforward, despite the presumed absence of gene flow. The geological histories of island systems are often complex and

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Data from molecular and morphological analyses are available via the Natural History Museum Data Portal (http://data.nhm.ac.uk).

Published online 16 Feb 2018

uncertain, and are not necessarily coincident with biotic histories. Moreover, various factors contribute to differences in relative rates of molecular and phenotypic evolution between insular and mainland populations (Millien 2006). For example, founder effects tend to reduce genetic variation within insular populations, yet inbreeding depression could manifest as developmental instability that increases variation or asymmetry of phenotypic traits (White and Searle 2008; Băncilă et al. 2010). Reduced population sizes on small islands hinder the ability of selection to purge mildly deleterious (i.e. nearly neutral) mutations from the genome, increasing rates to fixation of non-synonymous substitutions (Woolfit and Bromham 2005). Furthermore, island environments are presumed to be harsh and stochastic, selecting phenotypes that optimise energy efficiency (McNab 1994, 2002; Raia et al. 2010), and often leading to rapid evolution in body size and life history traits (i.e. island 'syndromes'). Although the extent to which these processes are directly relevant to speciation is debatable, they together create a milieu rife with complex evolutionary dynamics.

The speckled rattlesnake (*Crotalus mitchellii*) species complex inhabits rocky regions of the Mohave and Sonoran deserts of western North America, including several nearshore islands off the coast of the Baja California peninsula (Meik 2016) (Figure 1). Until recently, one species with four subspecies was recognised, distinguished from other rattlesnakes primarily by the extreme fragmentation of head scales notable of this complex (Klauber 1936; Meik 2008). Two previously recognised subspecies are insular populations from the Gulf of California that are giant (*C. m. angelensis*) and dwarfed (*C.*

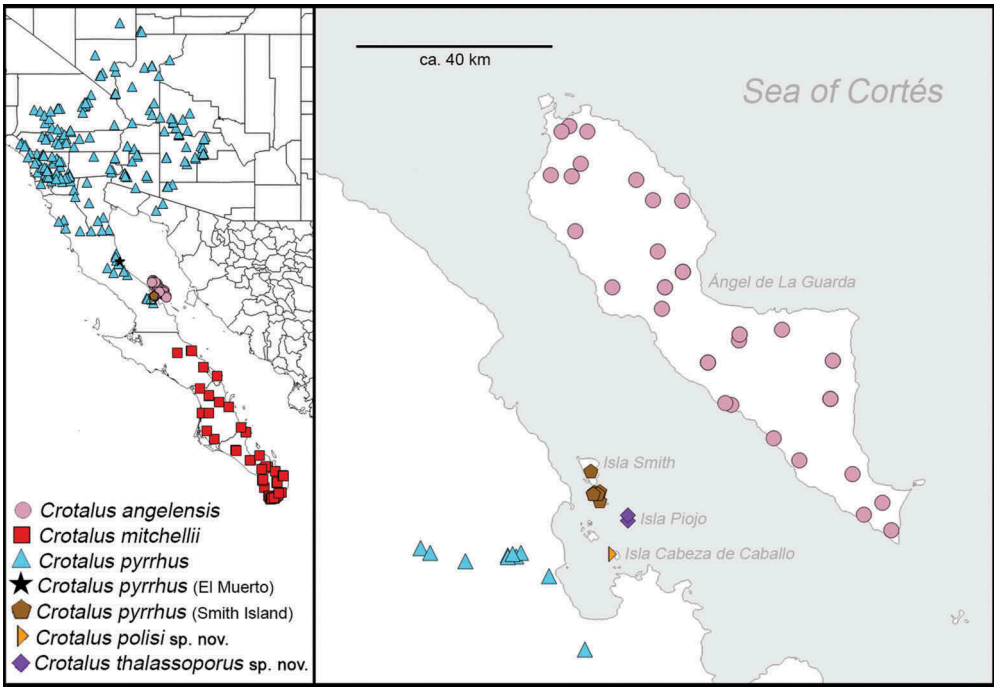

Figure 1. Distribution of species of the speckled rattlesnake (*Crotalus mitchellii*) species complex in western North America. Symbols represent sampling locations and indicate species and populations recognised in this publication.

m. muertensis) in body size, respectively. However, several other island populations are also diminutive in body size, but have not been recognised taxonomically (Meik et al. 2010, 2012a). In a recent systematic revision of speckled rattlesnakes that included molecular and morphological data for all subspecies, Meik et al. (2015) recognised three species within the complex: the south-western speckled rattlesnake (*Crotalus pyrrhus*), distributed from southern Utah and Nevada south to the mid-peninsular region of Baja California, including most coterminous island populations; the San Lucan speckled rattlesnake (*C. mitchellii*), occurring in the lower half of the Baja California peninsula, including all coterminous island populations; and the Angel de la Guarda Island speckled rattlesnake (*C. angelensis*), endemic to Angel de la Guarda Island in the central Gulf of California. In the same publication, *C. m. muertensis* was synonymised with *C. pyrrhus* because although somewhat dwarfed in body size, this island population was otherwise indistinguishable from mainland *C. pyrrhus* both phenotypically and genetically. However, Meik et al. (2015) also noted previously unrecognised divergence in other island populations, and posited that some of these might merit recognition as species. In particular, the population of dwarfed speckled rattlesnakes from Cabeza de Caballo Island constituted a deeply divergent mitochondrial clade sister to *C. angelensis*, and rendered *C. pyrrhus* paraphyletic at the mitochondrial ATPase 8 and 6 loci.

In this study, we expand on the previous work of Meik et al. (2015) to test hypotheses of species boundaries in various island populations of speckled rattlesnakes that have not been formally assessed taxonomically. In particular, we evaluate four populations of insular dwarfs that previously have been identified as phenotypically distinctive from adjacent mainland populations of *C. pyrrhus*: these populations originate from Cabeza de Caballo Island, El Muerto Island (described as *C. mitchellii muertensis* by Klauber 1949, and later relegated to *C. pyrrhus* by Meik et al. 2015), Piojo Island and Smith Island (Figure 1). We incorporated information from four intrinsic data classes, including three mtDNA gene sequences, an alignment of 6755 single nucleotide polymorphisms obtained from the nuclear genome (nSNPs), four microsatellite markers and 28 traits scored from external morphology. Collectively, these data sets allow for relatively fine resolution in population structure that could inform species limits. The results of our analyses indicate that species-level diversity is currently underestimated for this complex, and we herein describe two new insular species of diminutive rattlesnakes, adding further to our understanding of the exceptional biodiversity of the Gulf of California region.

Materials and methods

Species criteria

We view all multicellular organisms as constituting a multidimensional frequency distribution of peaks and troughs of relative cohesion in development, genomic architecture and other emergent properties that result from the collective history of shared reproductive opportunity and compatibility (i.e. processes operating through interacting replicators and meta-populations; see de Queiroz 2007; Hart 2010). Thus, we consider species as proxy labels that circumscribe patterns of biological variation among individual organisms. Species delimitation is the practice of testing whether collections of organisms correspond to these peaks of relative biological cohesion or similarity (i.e.

species hypotheses). Our view of species does not require absolute reproductive isolation or discrete diagnostic differences among groups, as we consider speciation a process with no distinct beginning or ending, and any temporal partitioning of organisms into species as they grade from ancestor to descendant lineages will be artificial (Godfrey-Smith 2014). Moreover, the circumscription of species boundaries will always require arbitrary decisions related to levels of divergence and the structure of variance, as variation is ubiquitous both within and among species, and estimates of species hypotheses likely will be vague on at least some axes of variation (Hendry et al. 2000; Hey 2006).

Here, we consider three primary criteria for evaluating hypotheses of species boundaries: (1) evolutionary history of putative lineages, (2) relative cohesion in multivariate variation across different biological attributes or axes (i.e. congruence of intrinsic biological cohesion patterns), and (3) comparisons with congeneric taxa that have been broadly accepted as adequate species on the basis of criteria (1) and (2) above. With respect to evolutionary history, it is widely accepted that individuals included in a species should be on average more closely related to each other than to members of other species (Kizirian and Donnelly 2004; Hey 2006), although we recognise that prior to lineage sorting, emergent species generally will render parental species non-monophyletic. We infer evolutionary history through gene trees, as hierarchical patterns of allele sorting can inform divergence and relative evolutionary independence among lineages. However, owing to population demographics and evolutionary processes, some genomic regions will not share the same history with other regions or with their multicellular hosts, which can confound inferences of species boundaries. To ameliorate these impacts on species delimitation, we evaluated evidence from different markers from both nuclear and mitochondrial genomes.

The rationale for the cohesion of multivariate variation criterion is based on the prediction that emerging species will eventually form genotypic and phenotypic clusters that are more or less distinct from other such clusters (Mallet 1995; Templeton 2006). Continuous quantitative characters or genetic data transformed into a quantitative data matrix should be normally distributed in biparental species that are close to Hardy–Weinberg equilibrium. Similarly, many meristic and qualitative characters follow an underlying normal distribution consistent with polygenic inheritance, but where traits are expressed by 'liability' thresholds (Wright 1934; Falconer and Mackay 1996; Lawing et al. 2008). Therefore, evidence for multiple normal distributions across multiple axes of variation (i.e. Gaussian mixed distributions) provides evidence for multiple species in a sample of comparable individuals (Ezard et al. 2010; Guillot et al. 2012; Zapata and Jimenez 2012).

Integrative taxonomy protocols

Integrative taxonomy incorporates various criteria and classes of empirical data for both discovering species entities and testing hypotheses of species limits (Padial et al. 2010; Miralles and Vences 2013; Pante et al. 2015). Although this total-evidence approach to species delimitation has a sound theoretical basis (Mayden 1997; de Queiroz 2007), the development of statistical methods that integrate different data classes into a common analytical framework has been slow (Sites and Marshall 2004; Yeates et al. 2011). Most

statistical approaches to phenotypic data analysis evaluate variation only once samples are *a priori* assigned to species, and are therefore of little use for discovering species boundaries. Similarly, most statistical approaches to inferring species trees also depend on *a priori* designation of samples to species (Carstens and Dewey 2010; O'Meara 2010; Carstens et al. 2013). However, recent computational advances have allowed for objective species discovery using model-based clustering, a method that algorithmically identifies numbers of natural clusters in a sample and assigns individuals to each using variance/covariance structures of multivariate data (Fraley and Raftery 2002; Ezard et al. 2010; Aguilar et al. 2016). Although inferred clusters could represent objective species hypotheses, statistically evaluating congruence of clusters inferred from different data classes has not been adequately addressed, and most studies continue to rely on arbitrary decisions as to what constitutes congruence.

In this study, we use an integrative taxonomy approach to test *a priori* hypotheses of species limits, and therefore bypass issues of species discovery. Our species hypotheses are based on previous discovery procedures (Meik et al. 2015), and the presumption that island populations of speckled rattlesnakes are likely isolated from each other and from mainland populations, and might have diverged sufficiently to represent distinct species. With respect to the evolutionary history criterion, Meik et al. (2015) used a single mtDNA gene fragment (ATPase 8 and 6) to construct a mitochondrial phylogeny for the *C. mitchellii* complex. Notably, this phylogeny had two deeply divergent clades of *C. mitchellii* (*sensu stricto*), which were rendered non-monophyletic by *C. stephensi* (although with little statistical support). In contrast, the nuclear phylogeny (based on 2318 concatenated SNPs) strongly supported the monophyly of the *C. mitchellii* complex (Meik et al. 2015, fig. 3). To further test the mitochondrial and nuclear monophyly of the *C. mitchellii* complex, we sequenced two additional mtDNA genes and added outgroups to the analysis, and also assembled a considerably larger nSNP data set. In addition to evaluating monophyly of the species complex, we used these molecular data sets to test the monophyly of hypothesised species within our delimitation scheme.

In order to explicitly test our species hypotheses in an integrative framework (i.e. multiple data classes), we developed a test that assesses the accuracy of identification of species within a single *a priori* species delimitation scheme. Our method is reminiscent of some recent species delimitation approaches that use Bayes Factors to rank different species hypotheses (Grummer et al. 2014; Leaché et al. 2014); however, unlike these methods (which exclusively use nuclear DNA), our method incorporates evidence across multiple data classes: (1) multivariate phenotypic data, (2) nuclear DNA microsatellites, (3) nuclear DNA SNPs, and (4) mitochondrial DNA sequence data. We first reduced dimensionality of each molecular data set using principal component analysis (PCA) of correlation matrices. We then applied linear discriminant analysis (LDA) with cross-validation to each reduced-dimension data set, and to the raw data matrix for phenotypic data, and then extracted posterior probabilities of assignment for each putative taxon. By averaging posterior probabilities across each data class and putative taxon, we were able to quantitatively assess the relative support for each species within our species delimitation scheme.

Data sets

Genomic DNA was isolated from muscle or liver tissue (preserved in ethanol or Sodium dodecyl sulfate-based lysis buffer) using standard blood and tissue extraction kits (QIAGEN, Valencia, CA). We PCR amplified (1) three mitochondrial gene fragments, 16S ribosomal subunit (16S), cytochrome *b* (cyt-b) and ATPase 8 and 6 (ATP), and (2) various nuclear microsatellite loci. Amplicons were generated using a variety of primers and thermal cycling profiles (Table 1). PCR reactions were purified using 1 µL of ExoSAP-IT (Affymetrix, Santa Clara, CA) for every 10 µL of amplified sample. For mitochondrial genes, BigDye terminator chemistry (ABI, Foster City, CA) was used to sequence both primer directions of the amplicon. Sequencing was conducted at the UTA Genomics Core Facility (Arlington, TX) and SeqWright DNA technology services (Houston, TX). Resulting chromatograms were cleaned and aligned using the program Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI). We screened 10 microsatellite primers developed for the closely related tiger rattlesnake, *C. tigris* (Goldberg et al. 2003; Munguia-Vega et al. 2009), and identified four that amplified across the *C. mitchellii* complex (Table 1). Allelic lengths were determined using 5′ fluorescently labelled primers (Integrated DNA Technologies, Coralville, IA) and an ABI 3100 series genetic analyser. We scored allelic lengths using the program GeneMarker (SoftGenetics, State College, PA).

Because rattlesnake specimens were collected from various islands during the same field expeditions, we confirmed patterns of mitochondrial diversity using a chain-of-custody test. For this procedure, we collected muscle tissue from formalin-fixed specimens accessioned at the University of Texas at Arlington Amphibian and Reptile Diversity Research Center (UTAARDRC) and Museo de Zoologia, Facultad de Ciencias, Universidad Nacional Autónoma de México (MZFC-UNAM) for which we also possessed DNAs obtained from fresh tissues. We soaked these tissues in a phosphate-buffered saline solution for 24 h, and then used a formalin-fixed paraffin-embedded DNA isolation kit (QIAGEN) to extract DNA. We then sequenced the mtDNA ATP fragment using protocols described above, and compared sequences to those obtained from fresh tissue samples taken in the field.

We reanalysed the RADseq data set of Meik et al. (2015) using Stacks 1.47 (Catchen et al. 2013). In order to maximise our SNP data for total evidence-based analyses, we also analysed the read 2 data (which Meik et al. [2015] had not done). We ran read 1 and read 2 data separately through quality filtering and Stacks 1.47 using the methodology described by Streicher et al. (2014). Briefly, this included implementing the following modules in Stacks 1.47: ustacks, cstacks, sstacks and populations. The 'populations' module was used to generate both SNP alignments (consisting of homozygous sites that varied across our sample) and genotypic tables (consisting of all homozygous and heterozygous sites). We concatenated both SNP alignments and genotypic tables by hand to combine read 1 and 2 data into a single input file. We allowed up to 50% missing individuals per SNP in all treatments.

We obtained a phenotypic data matrix of 28 external morphometric (nine linear measurements) and meristic (19 scale count and body pattern elements) characters for 372 subadult and adult specimens of *C. mitchellii, C. angelensis* and *C. pyrrhus*,

Table 1. Primers and polymerase chain reaction (PCR) conditions used to examine molecular variation in the speckled rattlesnake (*Crotalus mitchellii*) species complex.

					PCR conditions (°C)			
Genome	Locus	Direction	Sequence (5′ to 3′)	Reference	Denaturation	Annealing	Elongation	Cycles
mtDNA	16S	F	CGC CTG TTT ATC AAA AAC AT	Palumbi et al. (2002)	95 (30 s)	50 (30 s)	72 (2 min)	40
mtDNA	16S	R	CCG GTC TGA ACT CAG ATC ACG T		95 (30 s)	50 (30 s)	72 (2 min)	40
mtDNA	cyt-b	F	TGA CTT GAA RAA CCA YCG TTG	Palumbi et al. (2002)	95 (30 s)	45–48 (30 s)	72 (1 min)	30
mtDNA	cyt-b	R	CAT ATT AAA CCC GAA TGA TAY TT		95 (30 s)	45–48 (30 s)	72 (1 min)	30
mtDNA	ATPase	F	ATG CCA CAA CTA GAT ATT GTT	Meik et al. (2012b)	94 (30 s)	46 (30 s)	72 (1 min)	25
mtDNA	ATPase	R	CGG TGA TGT TGG CTG TAA GT		94 (30 s)	46 (30 s)	72 (1 min)	25
nDNA	Crti12	F*	6FAM-AGG AGT TAG GTA AGG AGT	Goldberg et al. (2003)	95 (30 s)	50 (30 s)	60 (1 m)	30
nDNA	Crti12	R	CAG ATA GAA AGC CAG CAA A		95 (30 s)	50 (30 s)	60 (1 m)	30
nDNA	Crti23	F*	HEX-GAT TGT GTG GTG TTT ATT GTT GC	Munguia-Vega et al. (2009)	95 (30 s)	50 (30 s)	60 (1 m)	30
nDNA	Crti23	R	GGA TGC TCC ATC ACT AGA CG		95 (30 s)	50 (30 s)	60 (1 m)	30
nDNA	Crti32	F*	6FAM- TCT AGT CAG CTC CTA GCA TTT TAA GG	Munguia-Vega et al. (2009)	95 (30 s)	50 (30 s)	60 (1 m)	30
nDNA	Crti32	R	GGT GTT TTC GGC TTC ATG C		95 (30 s)	50 (30 s)	60 (1 m)	30
nDNA	Crti47	F*	HEX-CAG GTG AAG GAG AAC TCT TGA TC	Munguia-Vega et al. (2009)	95 (30 s)	50 (30 s)	60 (1 m)	30
nDNA	Crti47	R	TCT TTG TAA TTG CTC CTC ATA TCC		95 (30 s)	50 (30 s)	60 (1 m)	30

including all relevant island populations. Details of the data set, including measurement protocols and museum voucher numbers, are provided in Meik et al. (2015).

Phylogenetic inference

To infer phylogenies from concatenated mtDNA and nSNPs, we used the program MEGA 6.06 (Tamura et al. 2013). The concatenated mtDNA alignment was 1806 total base pairs (bp), and nSNP alignment was 6755 bp (combined reads 1 and 2). We inferred phylogenies from both mtDNA and nSNP data sets using maximum likelihood analysis. Model selection for the mtDNA concatenated alignment resulted in the selection of the GTR + I + G model using MEGA 6.06. We applied a single GTR + G model to the nSNP alignment (*sensu* Cariou et al. 2013). We removed taxa with fewer than 200,000 sites (inferred in Stacks 1.47) from the nSNP analysis. Bootstrap support was obtained by summarising the results of 100 pseudoreplicates in the nSNP analysis. Nodal support for the mtDNA phylogeny was obtained from a partitioned Bayesian analysis in the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). We used the GTR + I + G model on five partitions (16S + ATP + codon positions in cyt-b) and ran the analysis for 1 million generations (sampling frequency 1000) summarising results with a burn-in of 100 generations. To determine average genetic distances among select mtDNA clades, we calculated average pairwise distances (dxy) in MEGA 6.06.

Population clustering

Our genotypic table comprised 29,624 nSNPs (combined reads 1 and 2). To evaluate whether species within our preferred delimitation scheme were close to Hardy–Weinberg equilibrium, we analysed our nSNP data using the program STRUCTURE (Pritchard et al. 2000). We ran the STRUCTURE analysis using K = 6 with the expectation that if nuclear DNA variation is matched to our delimitation scheme, then all individuals should be assigned to one of six distinct clusters with high probability. As with the nSNP phylogenetic analysis, we removed taxa with fewer than 200,000 sites (inferred in Stacks 1.47).

Linear discriminant analyses

We performed LDA on all four data sets. Discriminant analysis is an appropriate multivariate method for testing *a priori* hypotheses of species limits, as it incorporates empirical estimates of the variance/covariation structure for each putative species, which should correspond to unique distributions in multivariate space (Templeton 2006). LDA requires an *a priori* grouping variable (e.g. species delimitation scheme) and extracts underlying gradients of variation from sets of multivariate data (canonical functions) with the purpose of maximising variation among groups while minimising variation within groups along these extracted gradients (McGarigal et al. 2000). Canonical functions can then be used to evaluate relative cohesion among groups as

well as in a series of related procedures to probabilistically predict assignment of individuals to particular groups.

For genetic data sets, we first reduced dimensionality of raw data using PCA of correlation matrices, and retained the extracted composite variables (PCs) for input into LDA (i.e. discriminant analysis of principal components (DAPC); Jombart et al. 2010). As in Meik et al. (2015), we reduced the dimensionality of the mtDNA and nSNP data in the program adegenet 1.4 (Jombart and Ahmed 2011) using the fasta2genlight and read.structure commands, respectively. Missing data were then replaced using the na.replace command. We then performed LDA on scores from the first 10 PC axes. The first PC was strongly correlated with the amount of missing data for nSNP data ($R = 0.9624$, $P < 0.0001$, Spearman's Rho test). We therefore excluded PC 1 from analysis, and instead performed LDA on PCs 2–11 for the nSNP data set. To prepare the phenotypic data set for LDA, we first log-transformed all morphometric variables and then centred and scaled all variables to unit variance. We combined data from males and females for LDA, which allowed us to interpret results in the context of phenotypic variation among putative species inclusive of patterns of sexual dimorphism, thereby providing a more conservative assessment of divergence. We carried out LDA for each data set using the lda() function of the MASS package in R (Venables and Ripley 2002), with prior probabilities proportional to sample size. To measure the performance of each LDA model with respect to classification, we used a cross-validation procedure, which provides posterior probabilities of group membership for individual observations when they are excluded from the model. Finally, we generated a table of classification probabilities for each putative species across all four data sets based on the cross-validated classification means. In this way, we were able to quantitatively compare results of classification for putative species with well-established species of the *C. mitchellii* complex in an integrative framework.

Results

Phylogenetic relationships

Bayesian and maximum likelihood analyses of three concatenated mtDNA sequences strongly supported the mitochondrial monophyly of the *C. mitchellii* complex with *C. stephensi* as the sister taxon (Figure 2); however, one of two deep clades of *C. mitchellii* was moderately supported as sister to the remaining members of the complex, rendering *C. mitchellii* paraphyletic for this particular data set. Within populations recognised as *C. pyrrhus* and *C. angelensis* (including all insular populations), four major lineages were well supported, including a clade consisting of *C. angelensis* as sister to the Cabeza de Caballo population, a clade consisting of the remaining Baja California samples with shallow phylogenetic structure (including all other insular populations), a clade consisting of samples from most of the arid south-western United States with moderate structure (Arizona, Nevada, Utah and California), and a coastal California clade. Of the island populations, only *C. angelensis* and the population from Cabeza de Caballo Island were inferred as distinct and well-supported clades; all remaining insular samples showed low mitochondrial divergence from each other and the adjacent mainland samples (Figure 2, 'mixed haplogroup').

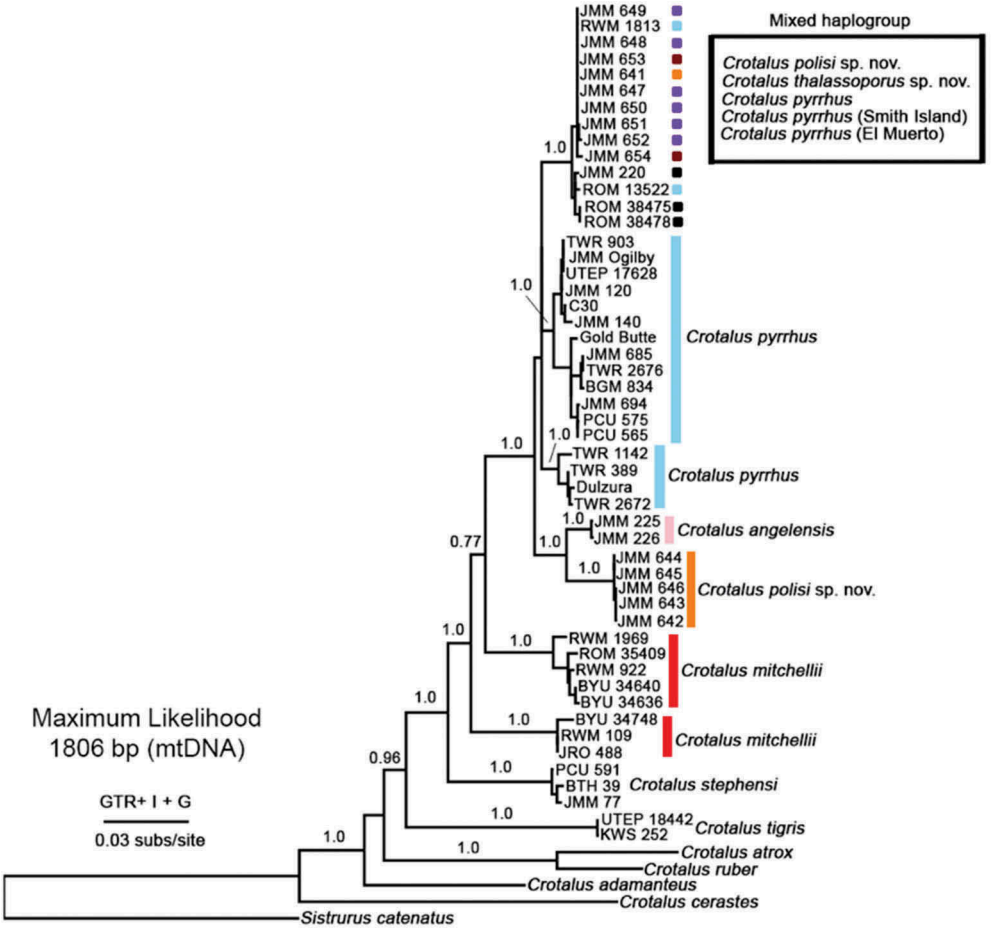

Figure 2. Maximum likelihood phylogram constructed from three concatenated mitochondrial gene fragments (ATP, cyt-b and 16S). Nodal support values are posterior probabilities from a partitioned Bayesian analysis. Populations or species discussed in the text are indicated with vertical bars.

As with the mtDNA phylogeny, the maximum likelihood analysis of 6755 nSNPs suggested a sister relationship between *C. angelensis* and the Cabeza de Caballo population (Figure 3); however, whereas this relationship was strongly supported in the mtDNA phylogeny, it was only weakly supported with nSNP data. Nonetheless, the Cabeza de Caballo population was itself strongly supported as a clade. The remaining insular populations (Smith and Piojo) were strongly supported as a clade, and this lineage was inferred as sister to mainland *C. pyrrhus*. However, within this insular clade, the Piojo Island population was strongly supported as a natural group whereas Smith Island was not. Within the mainland *C. pyrrhus* clade, the phylogeny showed successive branching with geographic structure from Baja California near the base of the tree through California, Arizona and Nevada at the most recent nodes, a pattern indicative of possible range expansion from south to north. The STRUCTURE analysis based on six groups (reflecting our species delimitation scheme) was mostly consistent

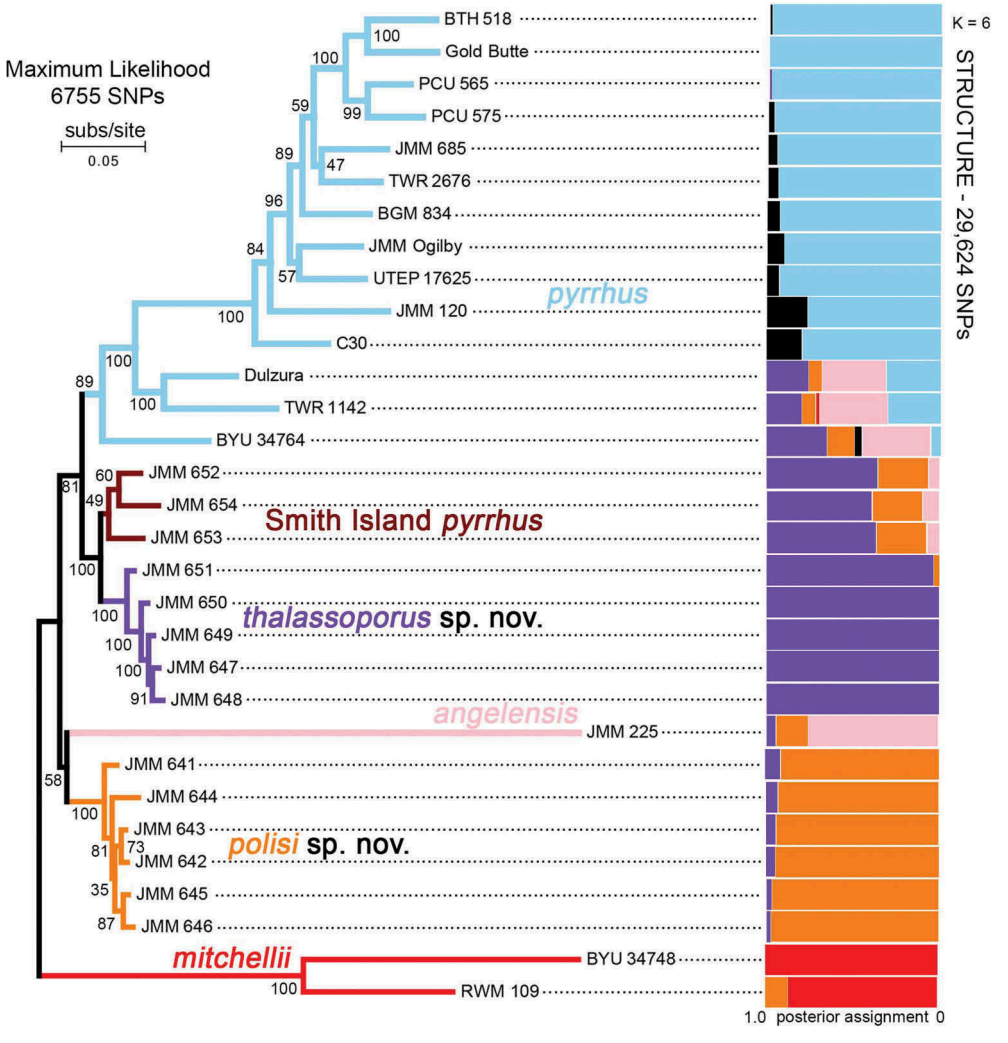

Figure 3. Maximum likelihood phylogram constructed from 6755 nSNPs (left), with nodal support derived from summarising results of 100 bootstrap pseudoreplicates, and posterior assignment of each individual to clusters using STRUCTURE (right), based on 29,624 biallelic nSNPs. Colours correspond to populations or species discussed in the text.

with major clades inferred from the nSNP phylogeny (Figure 3). Clusters based on posterior assignment of individual samples to groups corresponded mostly to *C. mitchellii, C. angelensis*, Cabeza de Caballo Island, mainland *C. pyrrhus* and Piojo Island. However, we also noted evidence of historical admixture among samples that cross most deeper nodes in the nSNP phylogeny. For example, the STRUCTURE analysis was equivocal in assignment of samples representing the basal branches within mainland *C. pyrrhus*, corresponding to Baja California and southern California. Similarly, there was also relatively high posterior assignment of Smith Island samples to both the Piojo and Cabeza de Caballo Island clusters.

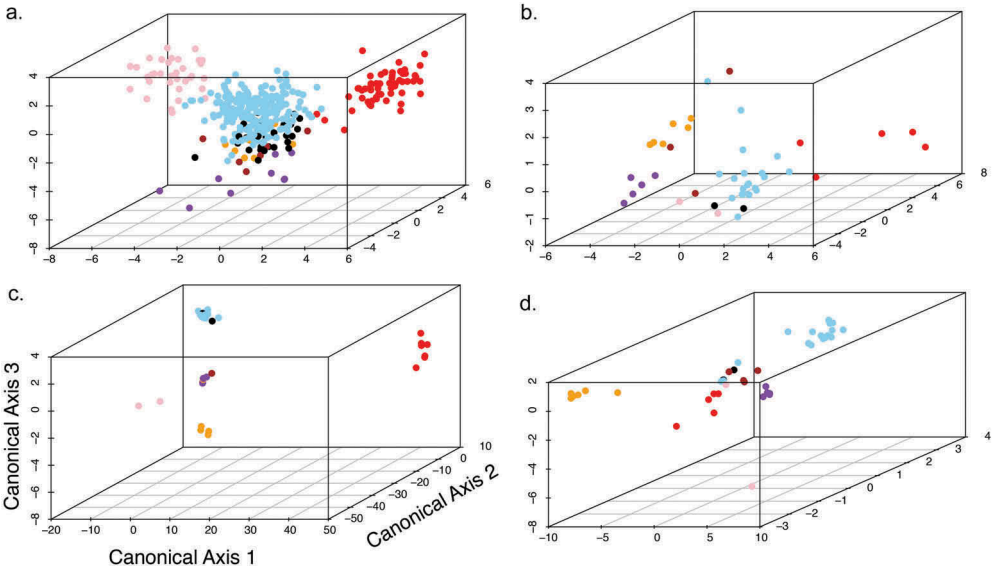

Figure 4. Scatterplots of first three axes from linear discriminant analyses of phenotypic data (a), principal component axes of microsatellite alleles (b), principal component axes of concatenated mitochondrial sequences (c), and principal component axes of concatenated nSNP data (d). Colour scheme: *Crotalus angelensis* = pink, *C. mitchellii* = red, *C. polisi* = gold, *C. pyrrhus* mainland = blue, *C. pyrrhus* El Muerto Island = black, *C. pyrrhus* Smith Island = brown, *C. thalassoporus* = purple.

Linear discriminant analyses

Results from LDA of the various data sets demonstrated that several putative species within our delimitation scheme formed distinct and cohesive groups in multivariate space defined by the first three canonical axes (Figure 4). Within-group clustering and between-group separation from the LDA of principal components was particularly evident for the mtDNA data set. The scatterplot of the first three canonical axes showed five distinct mitochondrial clusters corresponding to *C. mitchellii, C. angelensis*, Cabeza de Caballo Island, Piojo + Smith islands, and mainland *C. pyrrhus* + El Muerto Island (Figure 4c). With respect to the nSNP data set, the most cohesive and well-separated clusters were represented by Cabeza de Caballo Island, Piojo Island, most samples of mainland *C. pyrrhus*, and one of two individuals of *C. angelensis*, respectively. *Crotalus mitchellii* samples also formed a cohesive group, but this group was not well separated from a cluster consisting of the remaining samples, which included some mainland *C. pyrrhus* from Baja California and southern California, Smith Island, El Muerto Island, and the other *C. angelensis* specimen (Figure 4d). LDA of microsatellite data also showed a general pattern of samples grouping together within putative species, but with relatively little separation between groups. Samples from Smith Island did not form a cohesive cluster separate from *C. pyrrhus, C. angelensis* and Cabeza de Caballo populations, and the El Muerto samples were within a larger cluster that included *C. pyrrhus* and *C. angelensis* (Figure 4b). With respect to phenotypic data, LDA resulted in three distinct and mostly cohesive clusters consisting of *C. mitchellii, C. angelensis*, and *C. pyrrhus* + remaining insular populations (Figure 4a). The insular populations showed moderate

Table 2. Mean probability of assignment of individual samples to groups defined by our species hypotheses (i.e. established species within the *Crotalus mitchellii* complex and selected island populations from the Gulf of California, Mexico). Posterior probabilities are based on the cross-validation procedure of linear discriminant analysis (LDA) applied separately to each character set.

Species hypotheses	Morphology N = 372	mtDNA N = 46	Microsats N = 44	nDNA SNPs N = 43	Mean probability of assignment
C. angelensis	0.99 (N = 34)	1.0 (N = 2)	0.83 (N = 2)	0.62 (N = 2)	0.86
C. mitchellii	0.96 (N = 56)	1.0 (N = 8)	0.80 (N = 5)	0.74 (N = 6)	0.88
C. pyrrhus	0.95 (N = 224)	0.89 (N = 19)	0.88 (N = 20)	0.81 (N = 19)	0.88
Cabeza de Caballo Island	0.99 (N = 9)	0.83 (N = 6)	0.71 (N = 6)	1.0 (N = 6)	0.88
Smith Island	0.85 (N = 8)	0.15 (N = 2)	0.36 (N = 3)	1.0 (N = 2)	0.59
Piojo Island	0.89 (N = 9)	0.86 (N = 6)	0.94 (N = 6)	0.83 (N = 6)	0.88
El Muerto Island	0.72 (N = 32)	0.99 (N = 3)	0.52 (N = 2)	0.62 (N = 2)	0.71

disparity from mainland *C. pyrrhus* along the second and third canonical axes, but constituted a diffuse gradient cluster including all samples from the islands inhabited by dwarfed speckled rattlesnakes. We interpret this pattern as resulting from parallel evolution of these insular populations towards a dwarfed phenotype from an ancestral phenotype similar to that of mainland *C. pyrrhus* (see also Meik et al. 2010).

Posterior probabilities for group assignment using canonical functions ranged from 0.86 (*C. angelensis*) to 0.88 (*C. pyrrhus* mainland samples, *C. mitchellii*) for well-established species within the *C. mitchellii* complex (Table 2). By comparison, samples from both Cabeza de Caballo and Piojo islands had posterior probabilities of 0.88, suggesting that they are as assignable to their respective groups as are well-established species within the complex. In contrast, posterior probabilities for group assignment for Smith and El Muerto islands were considerably lower at 0.59 and 0.71, respectively. Overall, classification was more accurate for phenotypic data than for other data classes, and relatively low for microsatellites, for which we had only four loci.

Integrative taxonomy of speckled rattlesnakes

Based on our three criteria for recognising species applied across four classes of biological data, we find strong evidence for species recognition of the endemic population of speckled rattlesnakes on Cabeza de Caballo Island. With respect to evolutionary history, this taxon appears most closely related to *C. angelensis*, and is strongly supported as a clade based on both mitochondrial and nSNP trees. One individual possessing the endemic Cabeza de Caballo nuclear background had a mitochondrial haplotype that was distantly related to the remaining samples from the island, and instead was nested within a series of samples from Smith Island, Piojo Island, and *C. pyrrhus* from the Baja Peninsula. Therefore, we infer that reproductive isolation within the Cabeza de Caballo population is not complete. This mitochondrial haplotype likely arrived on Cabeza de Caballo as a recent immigrant from either the adjacent mainland population or from Smith Island, as indicated by the STRUCTURE plot (Figure 3). Results from LDA indicate that the Cabeza de Caballo population represents a distinctive and cohesive group for mtDNA, nSNP, and microsatellite data, and is also well separated phenotypically from *C. mitchellii* and *C. angelensis*

along the first three canonical axes. Phenotypic divergence between mainland *C. pyrrhus* and the four populations of insular dwarfs (including Cabeza de Caballo) was apparent along the second and third canonical axes, but with the exception of speckled rattlesnakes from Piojo Island, these populations were not strongly differentiated from each other (Figure 4A). However, based on LDA of the first 10 canonical axes, the Cabeza de Caballo population had a higher probability of assignment from phenotypic data than did the remaining taxa in our delimitation scheme (Table 2), suggesting that more relevant phenotypic variation on minor canonical axes helped discriminate this population from the other putative species.

We also found compelling evidence for species-level recognition of the endemic population of speckled rattlesnakes from Piojo Island. With respect to the mtDNA phylogeny, samples from this insular population were nested within a well-supported clade that included *C. pyrrhus* from the Baja Peninsula, El Muerto Island, Smith Island and a single specimen from Cabeza de Caballo Island (Figure 2). However, whereas specimens from El Muerto Island and *C. pyrrhus* from the peninsular mainland clustered together with *C. pyrrhus* from the western United States, individuals from Piojo and Smith islands were well separated from this group and clustered together in the scatterplot of canonical axes based on PCA of mtDNA data (Figure 4c). In contrast to results from mtDNA, the Piojo Island population was inferred as monophyletic from nSNP data, and also formed a cohesive cluster in the scatterplot of canonical axes based on PCA of nSNP data and microsatellites. Congruent with these patterns, the Piojo Island population also formed a mostly distinctive group in multivariate space based on phenotypic variation.

Based on total evidence, we consider the populations of speckled rattlesnakes from El Muerto and Smith islands to represent insular populations of *C. pyrrhus*. Although we were unable to include a sample from El Muerto Island in our expanded nSNP phylogeny, we previously demonstrated that this island population is unequivocally nested within a nSNP clade consisting of mainland *C. pyrrhus* (Meik et al. 2015, fig. 4). Furthermore, samples from El Muerto Island clustered with mainland *C. pyrrhus* across all data sets based on LDA, and had relatively low posterior assignment probabilities (Figure 4; Table 2). The population from Smith Island exhibited diffuse variation that possibly reflects the island's location in northern Los Angeles Bay close to the mainland, and a history of recurrent migration between it and both the mainland and the other Los Angeles Bay populations. Results from LDA of mtDNA, the nSNP phylogeny and the STRUCTURE plot collectively suggest a close affinity with the population from Piojo Island, which lies just to the south-east of Smith Island (Figure 1), whereas LDA of microsatellites and phenotypic data, and the mtDNA phylogeny all suggest a close affinity with samples from the peninsular mainland, Piojo, and Cabeza de Caballo islands. Based on these data and the low average posterior assignment of individuals to this insular population (Table 2), we consider rattlesnakes from Smith Island to represent a mongrel population that is not clearly on an evolutionary trajectory independent of *C. pyrrhus* or the other insular populations of speckled rattlesnakes.

Horsehead Island speckled rattlesnake

Crotalus ***polisi*** sp. nov.
(Figure 5; Table 3)

Crotalus mitchellii, Grismer 2002a, Murphy and Aguirre-Léon 2002, Meik et al. 2012a, in part. *Crotalus pyrrhus*, Meik et al. 2015, in part.

Description

A diminutive insular species of speckled rattlesnake with overall dusky appearance; background colouration is medium grey with a series of 36–48 indistinct and irregularly shaped dorsal body blotches, usually slate to charcoal grey in colour, only slightly darker than background; tail with 5–8 bands, last several are black with cream interspaces. The head is similar in colour to the body, with dark lateral suffusion and often with a faint postocular stripe and parietal blotches. Pattern is heavily punctated with black specks; blotches are usually wider than long, and merge with secondary lateral series to form muted crossbands on the posterior half of the body. A single row of nasorostral scales

Figure 5. Type localities and specimens photographed *in situ* for new species described herein. Piojo Island in foreground with Angel de la Guarda Island in the distance (upper left); western slope of Cabeza de Caballo Island in foreground with Ventana Island and peninsular mainland in the distance (upper right); paratype of *C. thalassoporus* (UTA R-59767) basking on a rock outcrop in the intertidal zone (lower left); holotype of *C. polisi* (MZFC-6248) coiled among boulders near the crest of the island (lower right).

Table 3. Selected phenotypic characters for species of the *Crotalus mitchellii* complex. For meristic characters, range is followed by mode, except for *C. polisi* and *C. thalassoporus*, where median is presented because of small sample sizes. For snout–vent length (SVL), range in mm is followed by average. Values for males are presented above those for females.

Character	*C. mitchellii* N = 56	*C. pyrrhus* N = 224	*C. angelensis* N = 34	*C. polisi* N = 9	*C. thalassoporus* N = 9
Prefrontals	23–46 (31)	20–54 (27)	21–34 (25)	24–32 (27.5)	13–20 (18)
	24–54 (30)	18–48 (34)	19–42 (26)	20–29 (21)	16–23 (20)
Interrictals	26–32 (28)	24–34 (29)	26–31 (30)	25–30 (26)	21–25 (22)
	25–33 (28)	25–32 (29)	25–31 (29)	23–26 (25)	23–25 (23)
Supralabials	14–17 (16)	13–19 (16)	12–16 (13)	13–15 (14)	13–16 (14)
	14–17 (16)	14–19 (16)	12–15 (13)	13–14 (14)	13–14 (14)
Infralabials	14–17 (15)	14–19 (16)	13–17 (15)	15–16 (15)	15–16 (16)
	13–17 (16)	13–19 (15)	12–17 (15)	12–15 (15)	15–16 (15)
Temporals	6–10 (8)	6–12 (8)	7–9 (8)	7–8 (7)	6–9 (7)
	7–10 (8)	7–10 (8)	6–10 (7)	7 (7)	7–8 (7)
Ventrals	160–178 (172)	165–187 (176)	180–186 (182)	163–173 (168)	164–175 (169.5)
	174–182 (180)	163–187 (176)	177–188 (188)	169–172 (169)	173–175 (174)
Subcaudals	22–29 (24)	20–27 (23)	23–29 (25)	21–24 (22.5)	21–24 (22)
	17–23 (20)	16–24 (19)	16–23 (20)	17–20 (17)	16–17 (16)
Rattle fringe	10–16 (12)	10–15 (12)	10–12 (10)	12–13 (12)	10–11 (10.5)
	12–15 (12)	10–14 (12)	10–12 (12)	10–12 (12)	11–12 (12)
Dorsal scale rows	23–27 (25)	21–27 (25)	25–27 (27)	21–23 (23)	21–23 (21.5)
	24–27 (25)	23–27 (25)	25–27 (27)	23 (23)	21–22 (22)
Dorsal body blotches	24–43 (32)	29–41 (34)	37–44 (40)	38–48 (42.5)	27–35 (32)
	27–41 (34)	26–41 (33)	35–45 (41)	36–40 (40)	31–40 (31)
Tail bands	3–6 (4)	4–8 (6)	5–8 (8)	5–8 (6)	4–5 (4)
	2–4 (3)	3–7 (4)	4–8 (5)	5 (5)	3–4 (3)
SVL	493–892 (736)	397–1106 (768)	528–1344 (947)	378–570 (499)	255–595 (466)
	394–889 (719)	401–957 (658)	492–1239 (757)	399–440 (419)	255–600 (405)

precludes contact between rostral and prenasal scales. Variation in selected phenotypic characters is presented in Table 3.

Diagnosis

The presence of nasorostral scales distinguishes the new species from all congeners except for species of the *C. mitchellii* complex. From *C. mitchellii* the new species differs in typically having more tail bands (range of mode or median between males and females of each species is presented for all comparisons: 5–6 vs 3–4), more dorsal body blotches (40–42.5 vs 32–34), fewer dorsal scale rows (23 vs 25), shorter ultimate supralabial scale (slightly longer than high vs twice as long as high), fewer supralabials (14 vs 16), fewer ventral scales in females (169 vs 180), fewer temporal scale rows (7 vs 8), colour pattern (mostly uniform colour pattern of slate or charcoal grey with indistinct blotches vs variable colour pattern), and smaller adult body size. From mainland populations of *C. pyrrhus* the new species differs in having typically more dorsal body blotches (40–42.5 vs 33–34), fewer dorsal scale rows (23 vs 25), fewer ventrals (168–169 vs 176), fewer temporal scale rows (7 vs 8), fewer supralabials (14 vs 16), colour pattern (mostly uniform colour pattern of slate or charcoal grey with indistinct blotches vs extremely variable), and smaller adult body size. From *C. angelensis* the new species differs in having typically fewer dorsal scale rows (23 vs 27), more supralabials (14 vs 13), fewer ventrals (168–169 vs 182–188), colour pattern (colour pattern of slate or charcoal grey with indistinct blotches vs buff or pink ground colour with grey to russet hexagonal

blotches), and smaller adult body size. From *C. thalassoporus*, the new species differs in having more tail bands (5–6 vs 3–4), more dorsal body blotches (40–42.5 vs 31–32), more interrictals (25–26 vs 22–23), more prefrontals (21–27.5 vs 18–20), and colour pattern (colour pattern of slate or charcoal grey with indistinct blotches vs fawn, pinkish or beige ground colour with indistinct rust-brown blotches).

Holotype

Adult female, MZFC-26408, field number JMM-642, collected on 18 March 2010 by Jesse M. Meik, Sarah Schaack and Matthew J. Ingrasci. Rostral plate slightly broader than high (2.5 × 2.2 mm). Head scalation highly irregular, making some scale designations ambiguous. Rostral-prenasal contact precluded by 3/3 nasorostral scales; two internasals contact rostral; distinct canthal scales absent, but approximately 29 knobby scales of variable size and shape in prefrontal area; interocular distance spans a minimum of six scales; loreal scales 4/4, irregularly shaped; preocular scales 2/2, upper prefoveal scales irregular, lower prefoveal scales large and broadly contact first three supralabial scales on both sides; subocular scales separated from supralabials by three scales at midpoint of eye; supralabial scales 14/14; infralabial scales 15/15; interrictal scales 25; dorsal scale rows at midbody 23; ventrals 168 (exclusive of one preventral); subcaudal scales 17, undivided; rattle fringe scales 12; rattle segments 6, button present.

Measurements. Snout–vent length (SVL), 445 mm; tail length, 23 mm; head length (rostral plate to articulation of mandible with quadrate), 20.7 mm; head width (at widest point just anterior to articulation with mandible), 19.9 mm; proximal rattle segment width, 8.5 mm.

Colouration and pattern in preservative. Head with diffuse black speckling; lateral surfaces of head dusky with faint postocular stripe; labial scales with cream blotches (appearing as 'blotch negatives' against surrounding ground colour); ventral surface of head cream with faint black specks on periphery, ventral surface of body also cream with diffuse black specks, becoming more prominent on posterior one fourth of body; 38 dusky grey body blotches with narrow (0.5–2 scales long) cream interspaces; blotches indistinct with ill-defined borders, all wider than long, fusing with faint lateral blotch series over posterior third of body to form crossbands; five tail bands, distal four black and only one to two scales long.

Type locality

Cabeza de Caballo Island, Municipality de Ensenada, Baja California, Mexico. Coordinates: N 28.971 W 113.479 (Figure 5).

Type deposition

Holotype at MZFC-UNAM; paratypes at MZFC-UNAM (MZFC 26407, MZFC 26409) and at UTAARDRC (UTA R-59763, UTA R-59764, UTA R-59765).

Etymology

The specific name is a patronym honouring the late Gary A. Polis of the University of California Davis, a renowned arachnologist and desert food-web ecologist, who died at

sea on 27 March 2000 when his research vessel capsised in a gale while returning to Bahía de Los Angeles from an expedition to Cabeza de Caballo Island. In addition to Polis, four other researchers, including postdoctoral fellow Michael D. Rose of UC Davis, and Takuya Abe, Masahiko Higashi and Shigero Nakano of Kyoto University, Japan, perished on that day. Four other UC Davis researchers and students survived the tragedy, and by their accounts, the deceased heroically gave their lives to help ensure their survival.

Louse Island speckled rattlesnake

Crotalus thalassoporus sp. nov.
(Figure 5; Table 3)

Crotalus mitchelli pyrrhus, Seib 1978, in part. *Crotalus mitchellii*, Grismer 2002b, Murphy and Aguirre-Léon 2002, Meik et al. 2012a, in part. *Crotalus pyrrhus*, Meik et al. 2015, in part.

Description

A diminutive insular species of speckled rattlesnake with overall pale appearance; background colouration tan to pinkish with a series of 27–40 indistinct blotches; blotches usually only slightly darker than ground colour, pinkish to pale brown, often appearing faintly rust-coloured; dark speckling on body is faint. Tail with 3–5 bands, last two or three distinctly black with pale cream interspaces. The head is similar in colouration to the body, but often with few dark specks, more conspicuous than stippling on trunk; grey postocular stripe indistinct. In colour pattern, *C. thalassoporus* is similar to *C. angelensis*, and in both species the anterior scales of the dorsal body blotches are tipped posteriorly with dark brown or black. A single row of nasorostral scales precludes contact between rostral and prenasal scales. Variation in standard phenotypic characters is presented in Table 3.

Diagnosis

The presence of nasorostral scales distinguishes the new species from all congeners except for species of the *C. mitchellii* complex. From *C. mitchellii* the new species differs in having typically fewer dorsal scale rows (21.5–22 vs 25), fewer subcaudals (16–22 vs 20–24), fewer temporal scale rows (7 vs 8), fewer supralabials (14 vs 16), fewer interrictals (22–23 vs 28), fewer prefrontals (18–20 vs 30–31), colour pattern (pale tan, pinkish, or beige ground colour with indistinct rust-brown blotches vs variable colour pattern), and smaller adult body size. From mainland populations of *C. pyrrhus* the new species differs in having typically fewer tail bands (3–4 vs 4–6), fewer dorsal scale rows (21.5–22 vs 25), fewer temporal scale rows (7 vs 8), fewer supralabials (14 vs 16), fewer interrictals (22–23 vs 29), fewer prefrontals (18–20 vs 27–34), colour pattern (pale tan, pinkish, or beige ground colour with indistinct rust-brown blotches vs extremely variable), and smaller adult body size. From *C. angelensis*, the new species differs in having typically fewer tail bands (3–4 vs 5–8), fewer dorsal body blotches (31–32 vs 40–41), fewer dorsal scale rows

(21.5–22 vs 27), fewer subcaudals (16–22 vs 20–25), fewer ventrals (169.5–174 vs 182–188), more supralabials (14 vs 13), fewer interrictals (22–23 vs 29–30), fewer prefrontals (18–20 vs 25–26), and smaller adult body size. From *C. polisi*, the new species differs in having typically fewer tail bands (3–4 vs 5–6), fewer dorsal body blotches (31–32 vs 40–42.5), fewer subcaudals in females (16 vs 17), more ventrals in females (174 vs 169), fewer interrictals in males (22 vs 26), fewer prefrontals (18–20 vs 21–27.5), and colour pattern (pale tan, pinkish or beige ground colour with indistinct rust-brown blotches vs slate or charcoal grey ground colour with indistinct blotches).

Holotype

Subadult male, MZFC-26410, field number JMM-648, collected on 19 March 2010 by Jesse M. Meik, Sarah Schaack and Matthew J. Ingrasci. Rostral plate is as high as broad (2.2 × 2.2 mm), separated from the prenasal scales by 3/2 nasorostral scales; internasal scales 2, with an additional tiny scale interpositioned at the anterior suture and in contact with dorsal edge of rostral. Head scalation highly irregular, making some scale designations ambiguous. Distinct canthal scales absent, but approximately 22 knobby scales of variable size and shape in prefrontal area; interocular scales 7; loreal scales 3/3, irregularly shaped; preocular scales 2/2, prefoveal scales 11/8, irregularly shaped, precluding contact between nasal scales and supraocular scales; subocular scales separated from supralabials by two scales at midpoint of eye; supralabial scales 15/15; infralabial scales 17/16; interrictal scales 24; dorsal scale rows at midbody 23; ventrals 169 (exclusive of three preventrals); subcaudal scales 21, undivided (except distal 3, which are divided); rattle fringe scales 10; rattle segments 3, chain incomplete.

Measurements. SVL, 341 mm; tail length, 28 mm; head length (rostral plate to articulation of mandible with quadrate), 18.5 mm; head width (at widest point just anterior to articulation with mandible), 15.7 mm; proximal rattle segment width, 6.7 mm.

Colouration and pattern in preservative. Overall ground colouration dirty cream with faint speckling; head with few faint grey specks; lateral surfaces of head with medium grey suffusion, labial scales with cream spots; ventral surface of head cream, immaculate, ventral surface of trunk cream with diffuse black specks; 37 indistinct body blotches only slightly darker than ground colour, some with pale centres, all primary dorsal blotches wider than long with exception of first four, fusing with faint lateral blotch series over posterior third of body to form crossbands; dark maculations on anterior and posterior margins of blotches give the impression of faint transverse bars along the length of the body; five tail bands, distal three black and two scales long.

Type locality

Piojo Island, Municipality de Ensenada, Baja California, Mexico. Coordinates: N 29.018 W 113.465 (Figure 5).

Table 4. Mean pairwise divergence across selected mitochondrial clades examined in this study (see Figure 2). Divergence is inferred from a concatenated alignment of three gene fragments (16S, cyt-b, ATP).

	Mixed haplogroup	*pyrrhus* 1	*pyrrhus* 2	*angelensis*	*polisi*
Mixed haplogroup (N = 14)	N/A				
pyrrhus 1 (N = 13)	0.021	N/A			
pyrrhus 2 (N = 4)	0.021	0.018	N/A		
angelensis (N = 2)	0.028	0.026	0.024	N/A	
polisi (N = 5)	0.038	0.033	0.032	0.023	N/A

Type deposition

Holotype at MZFC-UNAM; paratypes at MZFC-UNAM (MZFC 26411, MZFC 26412) and at UTAARDRC (UTA R-59766, UTA R-59767).

Etymology

The specific name is derived from the Greek word meaning 'seafarer', and is a reference to the apparent historical introgression we note between this taxon and the population of speckled rattlesnakes on Smith Island, most likely resulting from oversea dispersal of propagules from Piojo Island (see Discussion).

Discussion

Population divergence and biogeography of insular speckled rattlesnakes

Over the past 12 million years, rifting of the Pacific and North American tectonic plates formed the Baja Peninsula and associated Gulf of California (Lonsdale 1989; Ledesma-Vásquez and Carreño 2010). However, most of the details of this dynamic and complex geological history remain controversial (Hurtado et al. 2013), and much of the presumed history is based on *ad hoc* interpretations of phylogeographic patterns, often without corroborating geological evidence (Upton and Murphy 1997; Riddle et al. 2000; see also Grismer 2002a; Lindell et al. 2006; Leaché et al. 2007). The landmass that constitutes the present peninsula likely was sheered from the continental mainland as a series of fragments that drifted gradually towards the north-west, but whether these fragments split from the mainland as a series from north to south or from south to north is uncertain. Regardless, various proto-gulfs likely extended inland during this rifting process, many of which were transient or formed inland seas. Eventually, some of these seas coalesced into something similar to the modern Gulf of California between 5 and 3 million years ago (Carreño and Helenes 2002). In more recent times, excursions of the gulf extended far to the north of the present boundary into what is now the Colorado River Basin of the Sonoran Desert; the modern Salton Sea is a remnant of this ancient seaway. Some oceanic islands in the gulf were torn from the trailing edge of the peninsula as it approached its modern position, such as Angel de la Guarda Island, which is estimated to have been isolated from the peninsula for the past 2 to 1.5 million years (Carreño and Helenes 2002). Other islands in the gulf likely formed *de novo*, but most nearshore islands, including El Muerto and the islands of Los Angeles Bay, are land-bridge in origin, and formed between 14,000 and 9000 years ago as sea levels rose during the terminal Pleistocene and early Holocene (Wilcox 1978).

The geology of the mid-gulf region is particularly complex given that the islands of Los Angeles Bay share a recent terrestrial connection to the peninsular mainland, but also are in close proximity to the relatively ancient Angel de la Guarda Island, which is situated only 25 km to the north-east of Los Angeles Bay across the deep-water Ballenas Channel (Figure 1), and is part of an oceanic archipelago known as the Midriff Islands. The respective mitochondrial and nuclear genomes of insular speckled rattlesnake populations from this region reflect a correspondingly complex biotic history. In a previous study, we reported patterns of allele sorting consistent with introgression between *C. angelensis* and populations from the islands of Los Angeles Bay (Meik et al. 2015). With our expanded data set, we also infer parallel patterns of asymmetric introgression, where populations from the two outer islands of the system (Piojo and Angel de la Guarda islands) have influenced the molecular and phenotypic variation of rattlesnake populations from the respective inner islands (Smith and Cabeza de Caballo islands). However, whereas the patterns of apparent introgression between populations on Piojo and Smith islands are consistent with a Holocene history, the patterns of introgression between the populations on Angel de la Guarda and Cabeza de Caballo islands are more definitive, and suggestive of a deeper Pleistocene history.

As expected, the signature of introgressive hybridisation is relatively recent for *C. thalassoporus* and the population of *C. pyrrhus* on Smith Island, as both of these islands are land-bridge fragments that have been isolated only within the past 10,000 years. Piojo and Smith populations form a well-supported nSNP clade, and posterior assignment from STRUCTURE analysis supports a close nuclear genomic relationship between these populations. Similarly, the two populations cluster together in LDA of mitochondrial data, and are part of a shallow mitochondrial clade that includes all *C. pyrrhus* samples from peninsular Baja California. One scenario that could explain this pattern of association would be that Piojo and Smith islands continued to share a terrestrial connection for some time after these landmasses were isolated from the peninsular mainland, but based on strong support for the monophyly of the Piojo population but not for that of Smith Island, we suggest that a more parsimonious explanation is periodic overwater dispersal of propagules from Piojo Island to Smith Island.

In contrast to the observed pattern of shallow mitochondrial divergence expected from *C. thalassoporus* given the early Holocene history of Piojo Island, the deep mitochondrial divergence in *C. polisi* is enigmatic. From our previous analyses we inferred a deep and well-resolved mitochondrial sister clade for *C. angelensis* and *C. polisi*, and while our nSNP data hinted at this same arrangement, the phylogeny was unresolved (Meik et al. 2015, fig. 4). With additional data, we find further support for the sister-group relationship between *C. angelensis* and *C. polisi* from the perspectives of both nuclear and mitochondrial data, although this relationship remains weakly supported for nSNP data (Figures 2 and 3). However, given that Cabeza de Caballo is a land-bridge island of similar age to both Smith and Piojo islands, we find it difficult to reconcile the pattern of deep mitochondrial divergence in *C. polisi* with the recent geological history of the island, a history that is corroborated by the pattern of recent divergence in speckled rattlesnakes from neighbouring land-bridge islands influenced by the same Holocene climatic events.

Given the almost certainty that Angel de la Guarda and Cabeza de Caballo islands never shared a terrestrial connection, we perceive only two plausible explanations for the patterns of molecular variation in *C. polisi*. First, it is possible that Cabeza de Caballo is actually an oceanic island of much older origin than what has been generally accepted, and that migrants were exchanged between these islands during the early Pleistocene, eventually sorting into endemic haplogroups. Age estimates for land-bridge islands are based on extrapolating from sea channel depths and the eustatic sea level curve, and it is therefore possible that undetected changes in sea floor topography since the last glacial maximum could influence age estimates of islands. However, it is unlikely that such dramatic changes in sea floor topography could have occurred over such a short time period, and only adjacent to Cabeza de Caballo Island, without influencing the other islands of Los Angeles Bay.

An alternative hypothesis posits that Cabeza de Caballo is indeed a recent land-bridge island that harboured *C. pyrrhus* at the time of its isolation, but that early in the Holocene immigrants of *C. angelensis* arrived on the island, and as a result of introgressive hybridisation replaced the nuclear and mitochondrial haplogroups of the endemic population, after which the hybrid population experienced extraordinary mitochondrial substitution rate dynamics. Assuming that island age estimates are accurate, *C. angelensis* has been evolving in isolation for approximately 200 times the period that Cabeza de Caballo Island has been severed from the peninsular mainland, and observed mtDNA sequence divergences between *C. angelensis* and *C. pyrrhus* are roughly consistent with this time frame given a generalised vertebrate molecular clock of 2% per my for mtDNA (Klicka and Zink 1999) (Table 4). However, *C. polisi* is 2.3% divergent from *C. angelensis*, and is 3.8% divergent from *C. pyrrhus* and *C. thalassoporus* from the nearby islands and adjacent mainland (Table 4). A general expectation would be that speckled rattlesnakes on Cabeza de Caballo might have introgressed haplotypes from *C. angelensis*, but instead we observe a divergent and well-supported sister clade, indicative of considerable *in situ* molecular evolution in *C. polisi*. *Crotalus polisi* is even more divergent from mainland *C. pyrrhus* than is *C. angelensis*, and given the fraction of the maximum time of insular isolation for Cabeza de Caballo compared with Angel de la Guarda Island, this pattern can only be explained by rapid mitochondrial evolution. Our nuclear data set and phylogeny further supports this hypothesis in that *C. angelensis* has a long branch length consistent with the timeframe of isolation for Angel de la Guarda Island, whereas nuclear branch lengths are comparable between all three populations from islands in Los Angeles Bay, suggesting unremarkable nuclear evolution in *C. polisi* and a shared Holocene history of eustatic sea level change influencing these recent insular populations similarly. Furthermore, we suggest that the short internode linking the *C. angelensis*/*C. polisi* clade to the remaining populations of *C. pyrrhus*/*C. thalassoporus* and the low support for this group based on nSNP data could result from alleles retained from the ancestral *C. pyrrhus* population that was initially isolated on Cabeza de Caballo Island, confounding phylogenetic analyses.

Although we cannot confirm a specific mechanism for the signal of rapid evolution of the mitochondrial genome in *C. polisi*, we propose that this pattern might have been driven by unusual mitonuclear interactions (Levin et al. 2014; Wolff et al. 2014). Rapid changes in phenotype and behaviour are well documented among insular vertebrates (Gray et al. 2015; Hofman et al. 2015), and speckled rattlesnakes from Angel de la Guarda

Island and Cabeza de Caballo Island have undergone apparently rapid and opposing selection for body size, being the largest- and smallest-bodied rattlesnakes in the complex, respectively. At approximately 1 kg in mass, the average individual of *C. angelensis* is an order of magnitude heavier than the average individual of *C. polisi*, and the introduction of nuclear and mitochondrial genomes from a giant-bodied species already having evolved under insular conditions might have conferred initial selective advantage to the endemic population on Cabeza de Caballo, allowing for a genomic sweep. We envision that the same metabolic machinery encoded by coevolved mitochondrial and nuclear products in a giant rattlesnake might create a mitochondrial environment prone to oxidative stress and increased mutation rates in a dwarfed rattlesnake. Comparative molecular genomic studies and common garden physiological experiments would be necessary to evaluate whether mitonuclear interactions have influenced these enigmatic patterns of molecular evolution in *C. polisi*.

Inferring speciation from population divergence

As sequencing technologies have advanced, genomic data and multispecies coalescent models have increasingly been applied to species delimitation (Yang and Rannala 2010; Fujita et al. 2012; Harrington and Near 2012). The sheer volume of molecular data available from tools such as RADseq and the corresponding development of analytical methods now allow for remarkable resolution of population genetic structure. But this precision for resolving structure comes with the cost of poorly discriminating between intraspecific genetic structure and interspecific divergence. Consequently, genomic data used in the framework of multispecies coalescent models will tend to overestimate species boundaries, particularly in the absence of other types of data (Sukumaran and Knowles 2017). Moreover, because only 1–10% of the genome encodes protein products across most eukaryotes (Lynch 2007), such large-scale data sets will function as nets that capture predominantly neutral processes affecting molecular evolution, and therefore disproportionately will reflect population demographics over other processes occurring along the speciation continuum. Although traditional dogma encourages 'neutral' markers in species delimitation, speciation increasingly is viewed as resulting from the accumulation of genomic incompatibilities, and therefore should also emphasise adaptive shifts across emerging lineages (Van Valen 1976; Coyne and Orr 2004; Hausdorf 2011; Hill 2017).

For these reasons, integrative taxonomy that incorporates multiple data classes and considers divergence patterns along the continuum of speciation will remain the standard for robust species delimitation for the foreseeable future (see also Padial and de la Riva 2010; Carstens et al. 2013). However, increased resolution of population structure is not applicable to only genomic data, as statistical advances such as DAPC and model-based clustering have also facilitated fine-scale partitioning of biological variation with more limited molecular and phenotypic data sets, and this increased resolution might also have the undesired effect of confounding estimates of species boundaries with intraspecific substructure. In this study, we used as our primary species criteria the probability of assignment of specimens to hypothesised species based on four classes of data and comparisons with 'good' species. We believe this is a promising method for integrating different data sets for taxonomic evaluations, but from our empirical data we

also note two important caveats to this approach. First, discriminant analysis works best with accurate variance estimates, and therefore small sample sizes generally will lead to poorer discrimination owing to increased uncertainty in within-group variance. For example, *C. angelensis* is a distinctive taxon with respect to the nDNA SNP phylogeny (Figure 3), but had low posterior probability of assignment when these same data were applied to discriminant analysis, likely because of missing data and small sample sizes (Table 2). Second, our metric of average probability of assignment weights fundamentally different data sets equally, which means that discrimination based on only four microsatellite markers has the same weight as does a large nSNP data set. This decision on weighting was arbitrary and arguments could be made for weighted averages. Regardless, this criterion should affect all putative species equally and therefore should not hinder comparisons within a single study.

Our integrative taxonomic approach using different data classes allowed for a holistic perspective of divergence in speckled rattlesnakes. *Crotalus polisi* and *C. thalassoporus* are young species, whose emergence was influenced by sea level rise during the terminal Pleistocene; however, within only a few thousand years, these insular populations have diverged to the extent of having similar levels of diagnosability as do much older and widespread species of the *C. mitchellii* complex. Moreover, the reduction of many scale counts in both new species corresponds with insular dwarfism, and suggests that body size divergence is not simply a plastic response to insular conditions, but also entails a genetic component. In contrast, other populations of insular *C. pyrrhus* of similar age have not differentiated substantially enough to be recognised as species when multiple axes of variation are considered (e.g. Smith and El Muerto islands). As expected, mitochondrial variation in *C. thalassoporus* suggests a recent association with and little divergence from mainland *C. pyrrhus*, but this marker obscures the unique evolutionary history of this island population as revealed through phenotypic and nuclear data sets. In contrast, *C. polisi* has unexpectedly deep mitochondrial divergence, but aside from dwarfism shows somewhat less phenotypic differentiation than does *C. polisi*. Collectively, these results lead us to doubt the value of any single marker of species boundaries outside of the context of others, particularly for identifying recent species. From the perspective of taxonomy, the speckled rattlesnake complex has been challenging owing to conflicting patterns of variation. Although additional data have helped ameliorate these discordant signals, phylogenetic inferences likely have been plagued by various factors including introgressive hybridisation, contemporaneous speciation of older taxa leading to incomplete lineage sorting at deeper nodes in the phylogeny, rapid adaptive evolution on islands, and rapid lineage sorting in insular populations because of low effective population sizes when compared to the mainland populations.

Acknowledgements

For intrepid assistance in Baja California, we thank J. Reyes-Velasco, D. Dashevsky, C. Pyle, L. Cepko, M. Song, A.M. Lawing, C. Hernández-Jiménez, M. Ingrasci, C. Casola, K. Setser and E. Mociño-Deloya. For use of lab space and reagents, we are grateful to E. Smith and M. Fujita. For facilitation of permits and/or logistics in Mexico, we are indebted to J. Valdez-Villavicencio and M.I. Fuentes-Allen. We thank the following institutions for allowing access to preserved specimens: ASU (A.

Holycross), CAS (J. Vindum), IBH (G. Parra-Olea), KU (L. Trueb, R. Brown), LACM (R. Feeny, K. Beaman), MBM (A. Heindl), MVZ (J. McGuire, C. Spencer, C. Ciscero), UC (J. Knouft), MSB (T. Giermakowski), MZFC (A. Nieto, A. Hernandez), SDSNH (B. Hollingsworth, A. DeSoto), TAMU (J. Dixon and K. Vaughn), TNHC (T. LaDuc), UAZ (G. Bradley), UABC (J. Rodriguez, J. Valdez-Villavicencio), USNM (K. deQuerioz, R. McDiarmid), UTA (J. Campbell, C. Franklin) and UTEP (C. Lieb). We are grateful to the following individuals for providing tissue samples: K. Beaman, B. Hamilton, D. Lannutti, C. Lieb, B. Lowe, D. Mulcahy, R. Murphy and T. Reeder. Collecting permits for specimens obtained for this study were issued by the government of Mexico (SEMARNAT) to OFV (04769/09, 07266/12), and by the state of Arizona to JMM (M30207246). Research was carried out under University of Texas at Arlington IACUC no. A07.027. This work was supported by grants from T&E Inc., the Explorer's Club Exploration Fund, H. and L. Darley (Nancy Ruth Fund) and the T.I. Foundation, and Tarleton State University Office of Research and Innovation (JMM).

Disclosure statement

No potential conflict of interest was reported by the authors.

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