Short Communication

Hiding in the lianas of the tree of life: Molecular phylogenetics and species delimitation reveal considerable cryptic diversity of New World Vine Snakes

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ABSTRACT

The Brown Vine Snake, *Oxybelis aeneus*, is considered a single species despite the fact its distribution covers an estimated 10% of the Earth's land surface, inhabiting a variety of ecosystems throughout North, Central, and South America and is distributed across numerous biogeographic barriers. Here we assemble a multilocus molecular dataset (i.e. cyt *b*, ND4, cmos, PRLR) derived from Middle American populations to examine for the first time the evolutionary history of *Oxybelis* and test for evidence of cryptic lineages using Bayesian and maximum likelihood criteria. Our divergence time estimates suggest that *Oxybelis* diverged from its sister genus, *Leptophis*, approximately 20.5 million years ago (Ma) during the lower-Miocene. Additionally, our phylogenetic and species delimitation results suggest *O. aeneus* is likely a complex of species showing relatively deep species-level divergences initiated during the Pliocene. Finally, ancestral area reconstructions suggest a Central American origin and subsequent expansion into North and South America.

1. Introduction

New World vine snakes of the genus *Oxybelis* are long, slender snakes that possess a dramatically elongated head and are specialized for arboreality (Fig. 1). These species are diurnal with excellent vision for hunting small lizards. Currently there are four species of *Oxybelis,* all with populations in Central America (Köhler, 2008; Uetz et al., 2018). *Oxybelis wilsoni* Villa and McCranie, 1995 is known only from Isla de Roatán, Honduras while *O. brevirostris* (Cope, 1861) and *O. fulgidus* (Daudin, 1803) occur in Central and South America. However, the Brown Vine Snake, *O. aeneus* (Wagler, 1824) has a distribution far exceeding its congeners extending from southern Arizona southward through Central America and into South America to southeastern Brazil. This distribution covers more than 58° of latitude, a distance of more than 9000 km on a north-south axis, and approximately 15 million square kilometers (~10% of the Earth's land surface), making it one of the most widespread snake species on the planet (Keiser, 1982). *Oxybelis aeneus* has an elevational range extending from sea level to at least 2500 m and populations inhabit semi-desert, tropical savanna, seasonal deciduous forests, and tropical rainforest (Keiser, 1982; Van Devender et al., 1994). Due to the extensive distribution and morphological variation in *O. aeneus*, various populations were described as distinct numerous times (e.g. *Dryophis vittatus* Girard, 1854; *O. microphthalmus* Barbour and Amaral, 1926; *O. potosiensis* Taylor, 1941). However, in spite of the many geographic barriers (e.g. Isthmus of Tehuantepec, Isthmus of Panama, Mexican Volcanic Belt, Andes Mountains and the Amazon River) occurring across its range and morphological diversity, the Brown Vine Snake is currently considered a single, extremely variable species (Keiser, 1974, 1982).

Although *Oxybelis aeneus* and *O. fulgidus* have recently been included in broad molecular phylogenetic analyses to assess evolutionary histories among snake genera, the current phylogenetic position of *Oxybelis* within the Colubrinae is unresolved (e.g. Pyron et al., 2013; Figueroa et al., 2016) and relationships within the genus have not been investigated. In this study, we implement multiple phylogenetic analyses using mitochondrial (mtDNA) and nuclear (nDNA) DNA datasets to assess the evolutionary history and timing of diversification of the genus *Oxybelis*. More specifically, we conduct the first phylogeographic
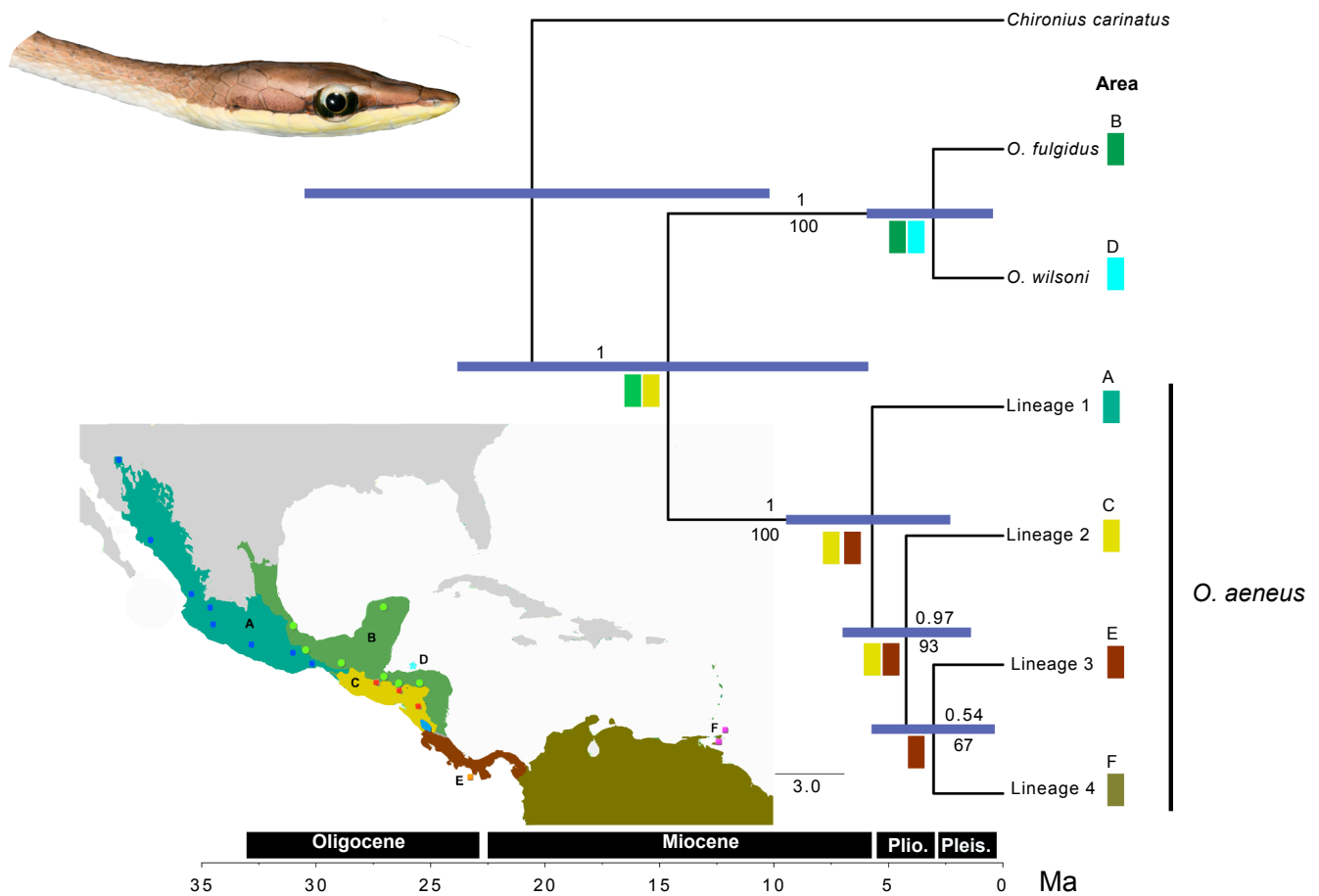
Fig. 1. Phylogenetic relationships and divergence times within *Oxybelis* based on coalescent-based species tree analysis in StarBEAST2 (cyt *b*, PRLR, cmos; total of 2112 bp). 'Species' were defined based on posterior probability support obtained from multiple BPP runs. Values above branch indicate posterior probability values for relationships, whereas values below nodes represent bootstrap support values from SVDquartets. Plio. = Pliocene; Pleis. = Pleistocene. Locality map representing populations of *Oxybelis* sampled (Table 1). Colored areas represent geographic regions used for DEC ancestral area reconstruction: (A) Sierra Madre Occidental Pine-oak forest, Sierra Madre del Sur Pine Forest, and Sinaloan Dry Forest (*O. aeneus Lineage 1*, sea green); (B) Central American Moist Forest (*O. fulgidus*, green); (C) Central American Montane Forest (*O. aeneus Lineage 2*, gold); (D) Isla de Roatán, Honduras (*O. wilsoni*, aqua star); (E) Isthmian-Pacific Moist Forest (*O. aeneus Lineage 3*, sepia); and (F) Trinidad and Tobago Moist Forest (*O. aeneus Lineage 4*, olive). Insert is an in-life photograph of *O. aeneus* from Tobago (J.C. Murphy). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assessment of the Brown Vine Snake, *O. aeneus*, throughout much of its northern range and assess if biogeographic barriers found to affect other Middle American taxa influence *Oxybelis*. Finally, we estimate ancestral areas to investigate the historical biogeography of *Oxybelis*.

2. Materials and methods

2.1. Molecular data

Full details for all methods and analyses are provided in the supplementary materials. Genomic DNA was extracted from tissues of *Oxybelis aeneus* and *O. fulgidus* (Table 1) using a Qiagen DNeasy extraction kit and protocol. Two mitochondrial [Cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 4 (ND4)] and two nuclear [oocyte maturation factor mos (cmos) and prolactin receptor (PRLR)] gene fragments were independently amplified using GoTaq Green master mix by Promega, (Madison, WI, USA) with the primer pairs: L14910 + H16064 (cyt *b*), ND4 + LEU (ND4), S77 + S78 (cmos), and PRLR_f1 + PRLR_r3 (PRLR). Annealing temperatures were 48, 46, 55, and 50 °C, respectively. Sequencing was performed in both forward and reverse directions using the PCR primers on a Beckman Coulter automated capillary sequencer, and sequence chromatographs were edited using Geneious R6 6.1.6. Gaps in alignments were treated as missing data and no internal stop codons were found. Novel sequences from this study were

deposited in GenBank (MK497173-MK497235) and combined with several other *Oxybelis* sequences, along with different colubrid sequences previously published on GenBank (Supplemental Appendix). PRLR sequences were generated in this study to assess intrageneric relationships within *Oxybelis* and were unavailable on GenBank for other taxa. Therefore this gene was not used for the analysis to determine the phylogenetic position of *Oxybelis* within the Colubridae. Likewise, ND4 sequences were generated in only a few *Oxybelis* specimens to evaluate the phylogenetic status of *Oxybelis* among numerous genera, for which ND4 sequences are prevalent, but was not sequenced in enough *Oxybelis* specimens to determine relationships within *Oxybelis* and therefore ND4 was excluded from those analyses. We selected ingroup taxa based on recent studies that found *Oxybelis* in a clade of New World colubrids (Pyron et al., 2013; Jadin et al., 2014; Figueroa et al., 2016).

2.2. Phylogenetic analyses

To determine the phylogenetic placement of *Oxybelis* within colubrids, we conducted three independent analyses using both maximum likelihood (ML) and Bayesian (BI) criteria. Both unpartitioned and partitioned (by locus) ML analyses of the concatenated cyt *b*, ND4, and cmos data (2319 bp) were performed in RAxML v.8.2.11 (Stamatakis, 2014) under a GTR + GAMMA model of nucleotide substitution (-f a

Table 1

Genbank numbers for DNA sequences analyzed in this study. Abbreviations of institutions and individuals for voucher specimens are as follows: ENS (Eric N. Smith field series), JAC (Jonathan A. Campbell field series), MSM (Mahmood Sasa Marín field series), MZFC (Museo de Zoología de la Facultad de Ciencias, Universidad Nacional Autónoma de México), UTA (Amphibian and Reptile Diversity Research Center, University of Texas, Arlington), USNM (Smithsonian National Museum of Natural History), and UWIZM (The University of the West Indies Zoology Museum). Additionally, ND4 is sequenced for UWIZM.2011.18.10 (Accession no. MK497235).

Species	Voucher	Locality	cyt *b*	cmos	PRLR
Oxybelis fulgidus	UTA R-52506	Veracruz, Mexico	MK497173	MK497197	MK497219
	UTA R-53002	Oaxaca, Mexico	MK497174	MK497198	MK497220
	JAC 24318	Chiapas, Mexico	MK497175	MK497199	MK497221
	UTA R-53417	Yucatan, Mexico	MK497176	MK497200	MK497222
	UTA R-45292	Izabal, Guatemala	MK497177	MK497201	MK497223
	MSM 439	Comayagua, Honduras	MK497178	MK497202	— — —
	USNM 565820	Olancho, Honduras	MK497179	MK497203	MK497224
O. aeneus Lineage 1	JAC 30618	Sinaloa, Mexico	— — —	MK497204	MK497225
	UTA R-53331	Nayarit, Mexico	MK497180	MK497205	MK497226
	UTA R-53373	Jalisco, Mexico	MK497181	MK497206	MK497227
	UTA R-57658	Colima, Mexico	MK497182	— — —	— — —
	UTA R-57659	Colima, Mexico	MK497183	MK497207	— — —
	UTA R-53374	Jalisco, Mexico	MK497184	MK497208	MK497228
	MZFC 19224	Guerrero, Mexico	MK497185	MK497209	MK497229
	UTA R-53024	Guerrero, Mexico	MK497186	MK497210	MK497230
	UTA R-53026	Oaxaca, Mexico	MK497187	— — —	— — —
	UTA R-52648	Oaxaca, Mexico	MK497188	MK497211	MK497231
Lineage 2	UTA R-46846	Zacapa, Guatemala	MK497189	MK497212	— — —
	UTA R-46865	Comayagua, Honduras	MK497190	MK497213	MK497232
	UTA R-44838	Jinotega, Nicaragua	MK497191	— — —	MK497233
Lineage 3	ENS 11259	Isla de Coiba, Panama	MK497192	MK497214	MK497234
Lineage 4	UWIZM.2011.18.10	Tobago	MK497193	MK497215	— — —
	UWIZM.2012.27.49	Tobago	MK497194	MK497216	— — —
	UWIZM.2011.20.14	Trinidad	MK497195	MK497217	— — —
	UWIZM.2016.23.5	Trinidad	MK497196	MK497218	— — —

search). Bayesian mixed-model analyses were performed in MrBayes v.3.0b4 (Ronquist and Huelsenbeck, 2003) using models selected based on Akaike information criterion (AIC) conducted in MrModeltest 2.2 (Nylander, 2004) run in PAUP*v4.0b10 (Swofford, 2002) (Supplemental Table S1). Two simultaneous runs were conducted (three heated and one cold chain with the default Markov chain Monte Carlo [MCMC] settings), for a total of 8×10^6 generations per run, sampling trees and parameters every 100 generations and the first 2×10^6 generations from each run were discarded as burn-in. Trees were rooted with close outgroup taxa *Grayia tholloni, Scaphiodontophis annulatus* and *Storeria dekayi* (Jadin et al., 2014). We used BEAST v.2.5.0 (Bouckaert et al., 2014) to estimate phylogenetic relationships and divergence times on the full matrix of 50 taxa. For temporal calibration we followed previous studies that used similar taxa (Burbrink and Lawson, 2007; Burbrink et al., 2008).

We utilized a taxonomically reduced multilocus data set consisting of partial sequences of cyt *b*, PRLR, and cmos (2112 bp) to examine the phylogeographic history of *Oxybelis*. We performed a ML analysis of the cyt *b* data only in RAxML and concatenated, partitioned ML and Bayesian analyses of all three loci. Trees were rooted with the close outgroup *Chironius carinatus* (Jadin et al., 2014).

Multiple coalescent-based phylogenetic and species delimitation analyses were performed. We used BPP v.4.0 (Yang, 2010; Yang and Rannala, 2014) to perform joint species delimitation and species tree inference (i.e. unguided species delimitation) using all three loci. Analyses were performed both with and without the mtDNA data to quantify concordance. Alignments included *Chironius carinatus* as the sole outgroup. We then used StarBEAST2 v.0.14.1 (Ogilvie et al., 2017) in BEAST v.2.5.0 (Bouckaert et al., 2014) to estimate species trees and divergence times of *Oxybelis*. Assignment of individuals to 'species' followed the results from BPP analyses. A total of seven species were defined, including the outgroup (*Chironius carinatus*). We calibrated the root node representing the divergence of *Oxybelis* from *Chironius* to estimate divergence times and substitution rates. Finally, we used

SVDquartets (Chifman and Kubatko, 2014) in PAUP* v.4.0a (Swofford, 2002) for coalescent-based species tree inference using the concatenated matrix. Individuals were assigned to the same 'species' as in the BPP and StarBEAST2 analyses. All quartets were evaluated and support for nodes was inferred using 100 nonparametric bootstrap replicates. Trees were rooted using *Chironius carinatus*.

We used RASP v. 4.0 (Yu et al., 2015) to estimate ancestral areas on the StarBEAST2 species trees. Inferences were based on multiple trees to explicitly account for phylogenetic uncertainty. The study area was divided into six contiguous non-overlapping ranges for analysis. Both unconstrained and temporally constrained statistical dispersal-extinction-cladogenesis (S-DEC) analyses were performed.

3. Results and discussion

3.1. Phylogenetic position of Oxybelis

Bayesian and ML analyses of the colubrid data set yielded moderate to strong support for the majority of nodes, particularly toward the tips of the tree (Fig. S1). Phylogenetic relationships among the analyses were similar with conflicts on poorly supported nodes. Examples of disagreements were the position of *Drymarchon corais* and the *Spilotes–Phrynonax* clade. Our study shows moderate support for a sister relationship between *Oxybelis* and *Leptophis* (Fig. S1) found in Figueroa et al. (2016), which is contrary to findings of Pyron et al. (2011, 2013) that show a sister relationship between *Oxybelis* and *Opheodrys*. More specifically, our results indicate that *Oxybelis* diverged from *Leptophis* in the lower-Miocene approximately 20 Ma. Support for relationships deeper in the tree were weaker; however, a *Chironius–Dendrophidion–Drymobius–Leptophis–Opheodrys–Oxybelis* clade recovered in Jadin et al. (2014) was strongly supported (0.97 posterior probability) in our BEAST analysis and weakly support in all ML and BI analyses.

3.2. Species diversity within Oxybelis

Our BPP results based on assigning individuals to lineages using the concatenated genealogy were highly congruent among runs and algorithms, indicating that the analyses were run for a sufficient duration (Supplemental Table S2). Results showed moderate to strong support for the majority of lineages (Fig. 1). Across runs and algorithms, the average posterior probability for a delimitation model consisting of seven species was 0.89. The only other model with some support was a model merging *O. fulgidus* and *O. wilsoni* (mean posterior probability = 0.10). All other delimitations received negligible support. Posterior probability values for four distinct 'species' within the currently defined *O. aeneus* were all > 0.99. We also conducted multiple BPP runs using only the nDNA to compare to the full data set. The highest posterior probability was for a model consisting of six species (average posterior = 0.72129). For these models, *O. fulgidus* and *O. wilsoni* were lumped into a single species. There was considerably less support for models containing five species (average posterior = 0.17162) and seven species (average posterior = 0.10623). Thus, the mtDNA data appear to provide additional evidence for the genealogical distinctiveness of *O. fulgidus* and *O. wilsoni*.

Using a seven species model (including the *Chironius carinatus* outgroup), we performed coalescent-based species tree inference in BPP using all three loci. The two independent BPP runs yielded an identical majority-rule consensus tree with strong support for several nodes. The topology was similar to the concatenated ML and Bayesian trees. The only difference was that BPP placed *Lineage 4* as sister to *Lineage 2*, whereas concatenated BI and ML placed *Lineage 3* as sister to *Lineage 4* (Figs. 1 and 2).

Because our BPP results indicated highest support for seven *Oxybelis* lineages (including the outgroup), we assigned each individual to its respective lineage for coalescent-based species tree inference using

StarBEAST2. StarBEAST2 runs indicated adequate mixing, stationarity, and ESS values (> 200) based on analysis in TRACER. The estimated mean substitution rate for cyt *b* was 9.2808E−3 [4.3502E−3 − 0.0158], the mean rate for PRLR was 6.0086E−4 [2.1247E−4 − 1.1444E−3], and the mean rate for cmos was 1.551E−4 [4.7532E−5 − 2.9885E−4]. The MCC tree recovered a topology identical to the concatenated ML and MrBayes trees. Posterior probability values were high for all nodes (> 95) except for the node uniting *Lineage 3* and *Lineage 4* as sister taxa (0.54). The species tree inferred by SVDquartets was also identical to the trees inferred using concatenated ML and Bayesian analyses and coalescent analysis in StarBEAST2. Bootstrap support (BS) for nodes was high, except for moderate support (BS = 67) for a sister relationship between *Lineage 3* and *Lineage 4*.

3.3. Evolutionary history within Oxybelis

The inferred phylogeographic history of *Oxybelis* was similar among our Bayesian and ML trees resulting from the cyt *b* only and multilocus data sets. Within *Oxybelis* we found strong support for two clades, those whose body colors are typically brown (i.e. *O. aeneus* sensu Keiser, 1974) and those that are green (e.g. *O. fulgidus* and *O. wilsoni*), which diverged during the mid-Miocene approximately 14.5 Ma (Figs. 1 and 2, Table 2). Further splitting within each lineage occurred more recently during the Pliocene with *O. fulgidus* and *O. wilsoni* diverging ca. 3 Ma and the four lineages within *O. aeneus* diverging between 5.7 and 3 Ma. Our results show genetic distinctiveness suggestive of multiple species-level lineages within *O. aeneus*.

Our study supports congruent lineage divergence events at biogeographic barriers similar to other Neotropical snake taxa. Specifically, *Lineage 1* diverged from the rest of the brown vine snakes approximately 5.7 Ma and this matches with divergences within populations of the Middle American rattlesnake *Crotalus simus* at the Isthmus of
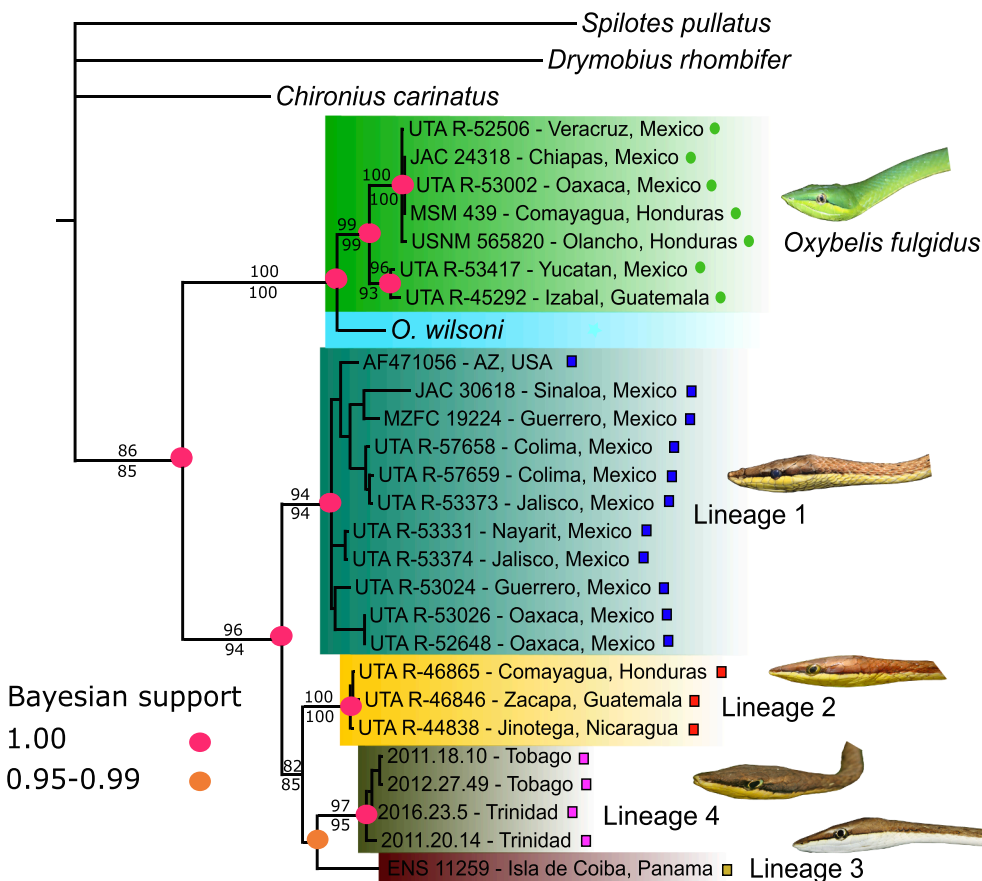

Fig. 2. Genealogical relationships within *Oxybelis* estimated from a Bayesian 50% majority-rule consensus phylogram of concatenated mtDNA (cyt *b*) and nDNA (PRLR and cmos) datasets (total of 2112 bp) with posterior probabilities (≥95) represented at the node. Additional bootstrap support values (≥70) from analyses using maximum likelihood from concatenated (above) cyt *b* only (below) datasets were included on branches. Some support values within clades are not shown due to space constraints. Symbols and clade colors on phylogeny correlate with locality symbols and areas covered in Fig. 1. In-life photographs are: *O. fulgidus* (Lake Miraflores, Selva Lacandona, Chiapas, Mexico, J. Reyes-Velasco), *O. aeneus* from Arizona (J.C. Murphy), *O. aeneus* from Mocorón, Gracias a Dios, Honduras (C.J. Franklin), *O. aeneus* from Tobago (J.C. Murphy), and *O. aeneus* from Panama (S. Lotzkat).

Table 2
Divergence time estimates (Ma) for significant splits within the *Oxybelis*. Mean values are given with corresponding lower and upper bounds of the 95% credibility for each estimate.

Node	Mean	Lower	Upper
Leptophis-Oxybelis	20.5929	12.5052	29.151
O. fulgidus-O. aeneus	14.6279	6.0306	23.6804
O. fulgidus-O. wilsoni	3.0422	0.5819	5.792
Lineage 1-Lineage 2	5.7099	2.4454	9.3173
Lineage 2-Lineage 4	4.2297	1.551	6.854
Lineage 3-Lineage 4	3.0255	0.5094	5.5872

Tehuantepec (Daza et al., 2010) or perhaps more likely breaks within *Atropoides*, *Bothriechis*, and *Cerrophidion* at the Motagua–Polochic Faults (Castoe et al., 2009). Furthermore, the split between the Northern Central American clade, *Lineage 2*, and the southern clade, *Lineages 3* and *4*, approximately 4.2 Ma, matches well with the separation between *Cerrophidion sasai* and *C. wilsoni* at the Nicaraguan depression (Castoe et al., 2009; Jadin et al., 2012).

Finally, our evolutionary analyses detected a pattern of divergence showing a Central American origin and expansion into North and South America (Fig. 1). Given that *Lineage 4* is the only South American lineage we have represented in our data and that it diverged from *Lineage 3* approximately 3.02 (0.5–5.58 Ma) it seems to support the Central American origin of the *O. aeneus* clade and its invasion of South America around the time the Panamanian isthmus closed, approximately 2.8 Ma (O'Dea et al., 2016). This pattern is supported by our ancestral area estimation that suggested an ancestral area encompassing southern Mexico and Central America for the genus as a whole (Fig. 1). A Central American origin was also inferred for *O. aeneus* (most likely western Central America; Area C) with more recent dispersal North (*Lineage 1*) and South (*Lineages 3* and *4*; Fig. 1). Ancestral area estimation using S-DEC in RASP yielded congruent results regardless of whether or not dispersal constraints were utilized. However, there was a fair degree of uncertainty in ancestral areas towards the root and we thus consider these results preliminary hypotheses that should be tested with broader sampling, particularly throughout South America and including *O. brevirostris*. Additional evidence for a Central American origin is that all known species of *Oxybelis* have distributions that center in Central America.

3.4. Future research

Our molecular analyses suggest there are four distinct lineages among populations currently described as *Oxybelis aeneus* (Figs. 1 and 2). We find this result remarkable considering our sampling of populations is limited to only the northern portion of its range. However, we wish to hold off on making formal taxonomic changes until additional specimens are examined from throughout the distribution and morphological data are collected. A project is currently underway to provide morphological support separating previously described taxa that have been synonymized along with description of new taxa (Jadin et al., in preparation). A more complete sampling of populations within the range of the *O. aeneus* complex should provide further insight into the biogeographic history of this clade and is likely to reveal more undescribed taxa.

Acknowledgments

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.01.022.

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