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Confirmation of introduced Louisiana pinesnakes, *Pituophis ruthveni*, in Florida based on molecular analyses

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***Abstract.*—As more wide-range phylogenetic studies are available, the opportunity arises to compare DNA from these data sets to suspected introduced individuals in order to confirm species identification and determine their geographic origins. Two recently collected *Pituophis* specimens in Miami-Dade County, Florida, were examined using molecular analyses. Maximum likelihood and Bayesian inference methods place our specimens within the *P. catenifer sayi* / *P. ruthveni* clade. Additional morphological evidence support their identification as the Louisiana pinesnake, *Pituophis ruthveni* Stull 1929, a species indigenous to a small area in western Louisiana and eastern Texas and candidate for listing by the U.S. Fish and Wildlife Service. Although *P. ruthveni* is viewed as a distinct species from *P. catenifer sayi* based on allopatry and differences in color pattern, no molecular evidence was found supporting the recognition of *P. ruthveni* as a separate species. However, adding other mtDNA and nuclear DNA genes might provide needed data for distinguishing between these two named taxa.**

Key words. DNA, exotics, ND4, mitochondrial, mtDNA, nonnative, phylogenetics, Squamata, species

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Introduction

Introduced species (e.g., stages 2–5 after Colautti and MacIsaac 2004) are those transferred from their native range into a new nonindigenous area. Over the past century it has become increasingly clear how disruptive human-caused biological introductions have been to the planet. While not all introduced species cause obvious harm, some introduced species can eventually become economic threats and lead to serious conservation problems (Simberloff et al. 1997). As of 2005, it was estimated that the cost of environmental damages, losses, and control due to introduced species exceeded $120 billion per year in the United States alone (Pimentel et al. 2005). Prior to 2011, the state of Florida had 137 documented introduced reptile and amphibian taxa (56 being established), which ranks highest in the world (Krysko et al. 2011a, 2012). Invasion pathways in Florida include (fewest to highest numbers) biological control, zoos, cargo/plant shipments, and the pet trade.

Pinesnakes, bullsnakes, and gophersnakes (*Pituophis* Holbrook 1842) are large (up to 254 cm total length) constrictors native to North America, characterized by disproportionately small heads, four prefrontal scales, and a large rostral plate that extends upwards between the internasals (Conant and Collins 1991). Based primarily on molecular data using Parsimony and Maximum Likelihood analyses with 893 base pairs (bp) of the nicotinamide adenine dinucleotide dehydrogenase subunit 4 (ND4) region (Rodriguez-Robles and De Jesus-Escobar 2000), the *P. melanoleucus* species complex contains three currently recognized species; *P. melanoleucus* (sensu stricto; Pinesnakes; with three subspecies *P. m. lodingi*, *P. m. melanoleucus*, *P. m. mugitus*), *P. catenifer* (gophersnakes and bullsnakes; with six subspecies *P. c. affinis*, *P. c. annectens*, *P. c. catenifer*, *P. c. deserti-*

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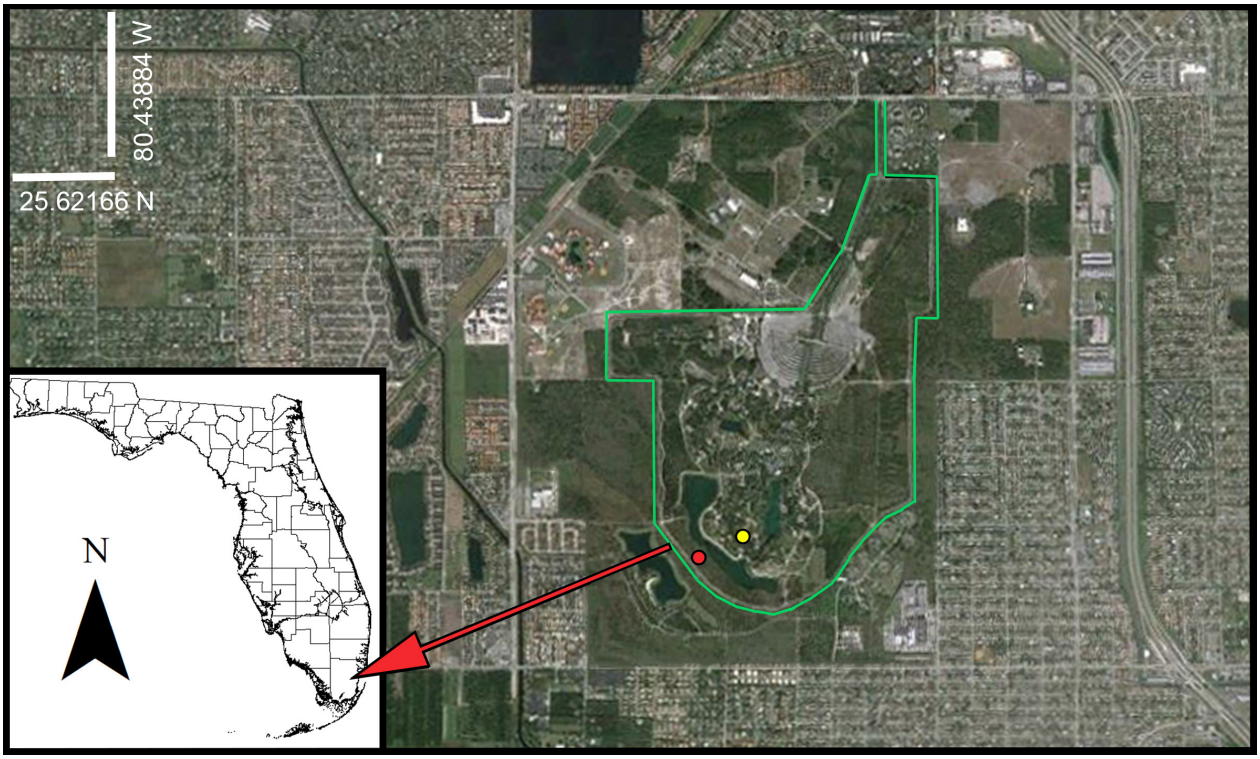

Figure 1. Map of Zoo Miami bounded in green. Note that major roadways, residential areas, and undeveloped protected lands surround zoo property. Dots represents locations of *Pituophis* found on zoo property; yellow = UF-Herpetology 157954 (gravid female) and red = UF-Herpetology 163092 (adult male).

cola, *P. c. pumilis*, *P. c. sayi*), and *P. ruthveni* (Louisiana pinesnake). *Pituophis melanoleucus* (Daudin 1803) occurs in the eastern United States from southern New Jersey south to extreme southern peninsular Florida (i.e., Miami-Dade County; Krysko et al. 2011b) and west to Kentucky and southeastern Louisiana (Rodriguez-Robles and De Jesus-Escobar 2000). This species lacks a dark line from the eye to the angle of the jaw, has a dorsal pattern either absent (uniform black), obscure, or whitish to brownish with 23–30 distinct dark dorsal body blotches that are clearly separated from each other both anteriorly and posteriorly along the body and tail (Knight 1986; Powell et al. 1998; Reichling 1995; Thomas et al. 1976). *Pituophis catenifer* occurs from the Pacific Ocean east to Wisconsin, Illinois, and Texas, and from Canada south to Mexico (Rodriguez-Robles and De Jesus-Escobar 2000; Powell et al. 1998). This species typically has a dark line from the eye to the angle of the jaw, and a yellow or cream-colored dorsal pattern with 41–79 distinct dark dorsal blotches that are clearly separated from each other both anteriorly and posteriorly along the body and tail (Knight 1986; Powell et al. 1998; Reichling 1995; Thomas et al. 1976). *Pituophis ruthveni* occurs in allopatric populations in western-central Louisiana to eastern Texas (Ealy et al. 2004; Powell et al. 1998). This species sometimes lacks a dark line from the eye to the angle of the jaw, and has a pale brown dorsal pattern with 28–38 dark dorsal blotches; near the head the blotches obscure the ground coloration, whereas near the tail they are distinctly separated from each other (Knight 1986; Powell et al. 1998; Reichling 1995; Stull 1929; Thomas et al. 1976). Although *P. ruthveni* is nested within a clade containing only *P. c. sayi*, it is recognized as a separate species because it occurs in allopatric populations and is somewhat diagnosable using color pattern characters (Collins 1991; Knight 1986; Reichling 1995; Rodriguez-Robles and De Jesus-Escobar 2000; Thomas et al. 1976). *Pituophis ruthveni* is also a candidate for listing as an imperiled species by the U.S. Fish and Wildlife Service (2013).

The last known *Pituophis melanoleucus* from extreme southern peninsular Florida (UF-Herpetology 45970) was collected in 1980 in a disturbed pineland (with *Casuarina* and *Schinus*) in Cutler Ridge, Miami-Dade County, and because of ongoing dense urbanization this species is believed to be extirpated along the Atlantic Coast Ridge (Krysko et al. 2011b). In 2010, two *Pituophis* were collected on the Atlantic Coast Ridge at Zoo Miami, Miami-Dade County; one was found in an undeveloped area and another near public access. Based on color pattern alone, these snakes were suspected to be introduced *P. ruthveni* and reported to represent the first known vouchers for this species in Florida (Krysko et al. 2011a). Many documented introductions categorize species based on sometimes vague superficial morphology, such as color patterns, which may or may not be arbitrary human constructs. However, as more wide-range phylogenetic studies are conducted and published, the opportunity arises for other researchers to compare DNA from known data sets to suspected introduced individuals in

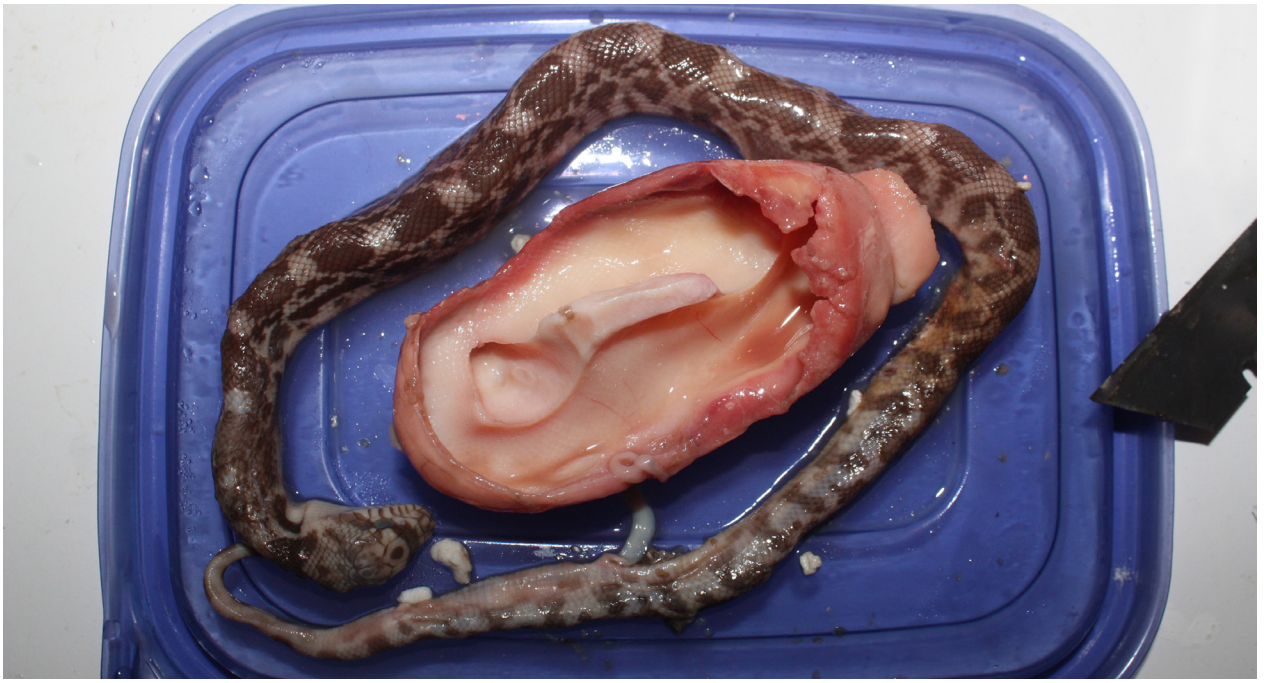

Figure 2. Well-developed *Pituophis* embryo (UF-Herpetology 164295) oviposited from wild collected gravid female (UF-Herpetology 157954) in Miami, Miami-Dade County, Florida.

order to confirm species identification as well as determine their geographic origins. In this paper, we conduct molecular analyses of *Pituophis* in a coalescent framework to confirm species identity and phylogenetic placement of our two specimens, followed by more detailed examination of morphology and color pattern.

Material and Methods

Site description and specimen acquisition

Zoo Miami is situated at 12400 SW 152th Street, Miami, Miami-Dade County, Florida, USA (Fig. 1; 25.611926°N, 80.398042°W, Datum WGS84, elev. 2 m). The property consists of ca. 300 ha, 106 ha of which are undeveloped managed lands, predominantly of pine rockland habitat. Zoo Miami property is surrounded by a mixture of natural areas, disturbed areas, and a county park, followed by dense urbanization.

On 16 May 2010 at 1645 h, an adult *Pituophis* (gravid female, 1,302 mm SVL, 1,486 mm TL; UF-Herpetology 157954; see Fig. 86 in Krysko et al. 2011) was collected in a service area behind a large animal exhibit (25.60395°N, 80.4006°W). This snake was observed by zoo staff the previous day along an adjacent public walkway, but was not captured. This snake was retained in captivity and oviposited three eggs on 22 June 2010. The eggs were viewed with a light on 28 June 2010; all three eggs contained a dark blood spot, but only one egg had an obvious network of veins developing. The first two eggs failed to develop and were discarded on 06 July 2010. The third egg had an unpleasant odor and was frozen on 14 September 2010; it was dissected on 20 September 2010 and revealed a well-developed embryo (UF-Herpetology 164295; Fig. 2).

On 25 December 2010 at 1215 h, another adult (male, 1,425 mm SVL, 1,635 mm TL) *Pituophis* (UF-Herpetology 163092) was collected in an undeveloped area (25.60304°N, 80.40295°W), across a large man-made lake and 0.26 km southwest of the first snake.

The well-developed embryo, shed skins from the two adults, and digital images were deposited in the Division of Herpetology, Florida Museum of Natural History, University of Florida. The female (UF-Herpetology 157954) is currently housed at the Memphis Zoo, and the male (UF-Herpetology 163092) is housed at Zoo Miami.

Laboratory techniques

DNA isolations were obtained using QIAquick PCR Purification Kit and DNeasy Blood and Tissue Kit (Qiagen Sciences, LLC). Using total cellular DNA as a template and Polymerase Chain Reaction (PCR) methodology (Saiki et al. 1988), mitochondrial DNA (mtDNA) was amplified and sequenced for the ND4 region following Rodriguez-Robles and De Jesus-Escobar (2000). The ND4 region includes a section of the 3' end of the ND4 gene, and two subsequent transfer ribonucleic acids ($tRNA^{His}$, $tRNA^{Ser}$), which were sequenced using the primers ND4 and Leu (Arevalo et al. 1994). PCR was conducted in 25 µl reactions: 9.5 µl H_2O, 12.5 µl GoTaq® Master Mix (Promega Corp, Madison, Wisconsin, USA), 1.0 µl each primer (10 µM), and 1.0 µl DNA template. PCR parameters included initial denaturing at 94 °C for three min, followed by 35 cycles of amplification: de-

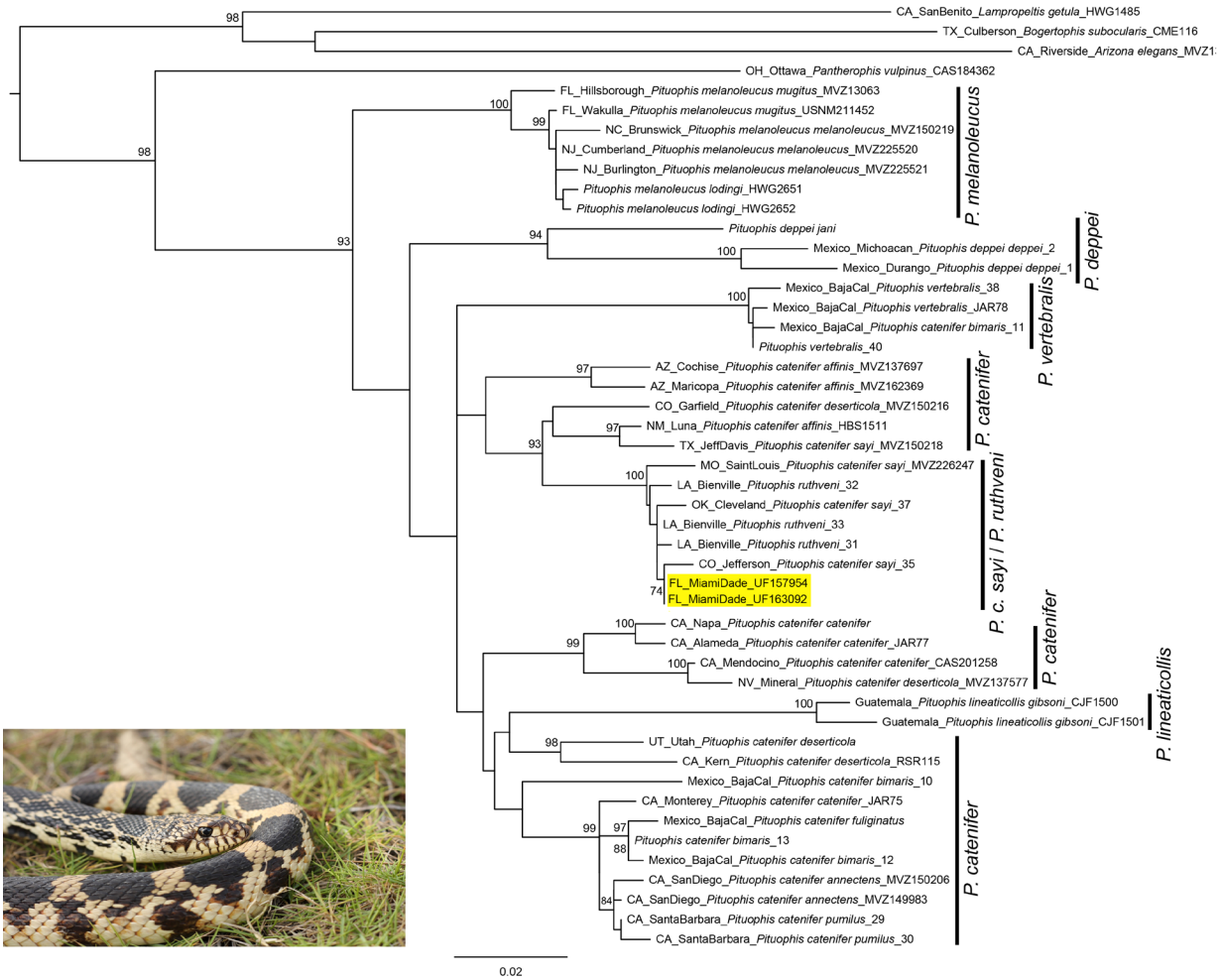

Figure 3. Maximum Likelihood phylogeny for *Pituophis* (Squamata: Colubridae) snakes, including the two known *P. ruthveni* (highlighted in yellow, UF-Herpetology 157954 and 163092) collected in Miami, Miami-Dade County, Florida. Note that values (≥ 50%) above nodes represent bootstrap support. *Inset photograph of UF-Herpetology 157954 by Dustin C. Smith.*

naturing at 94 °C for one min, annealing at 52 °C for one min, and extension at 72 °C for one min, followed by a final extension at 72 °C for seven min. Three µl of each PCR product were electrophoresed on a 1% agarose gel, visualized with GelRed™ staining (Biotium Inc., Hayward, California, USA), and compared with a DNA standard. Sequence files from the automated sequencer (Genomics Division, Interdisciplinary Center for Biotechnology Research, University of Florida) were assembled and edited as necessary with Geneious software (ver. 6.1, created by Biomatters. Available from http://www.geneious.com).

Phylogenetic analyses.—DNA sequence data were downloaded from GenBank for 46 snakes, including 42 *Pituophis*, and one of each *Lampropeltis getula*, *Pantherophis vulpinus*, *Bogertophis subocularis*, and *Arizona elegans* incorporating the original data set from Rodriguez-Robles and De Jesus-Escobar (2000) and current taxonomy after Pyron and Burbrink (2009). GenBank Accession numbers for our two *Pituophis* specimens (UF-Herpetology 157954 and 163092) are KJ938643 and KJ938644, respectively.

A total of 48 specimens with 875 base pairs (bp) of sequence data were analyzed. Relationships among mtDNA haplotypes were estimated using both Maximum Likelihood (ML) and Bayesian Inference (BI) methods. ML was conducted with the General Time Reversible model with gamma distributed rate heterogeneity (GTR + Γ) and 1,000 nonparametric bootstrap replicates (Felsenstein 2004) to assess node support using RAxML-HPC BlackBox (Stamatakis 2006; Stamatakis et al. 2008) from the CIPRES Science Gateway (Miller et al. 2010).

BI was conducted using BEAST (ver. 1.8; Drummond and Rambaut 2007) from the UF-HPC Galaxy instance (http://hpc.ufl.edu; Blankenberg et al. 2010; Giardine et al. 2005; Goecks et al. 2010). The Bayesian Information Criterion in jModelTest (ver. 2.1.4; Darriba et al. 2012; Guindon and Gascuel 2003) determined the best-fit nucleotide substitution model to be Hasegawa, Kishino, and Yano with a proportion of invariant sites and gamma distributed rate heterogeneity (HKY + I + Γ). A relaxed

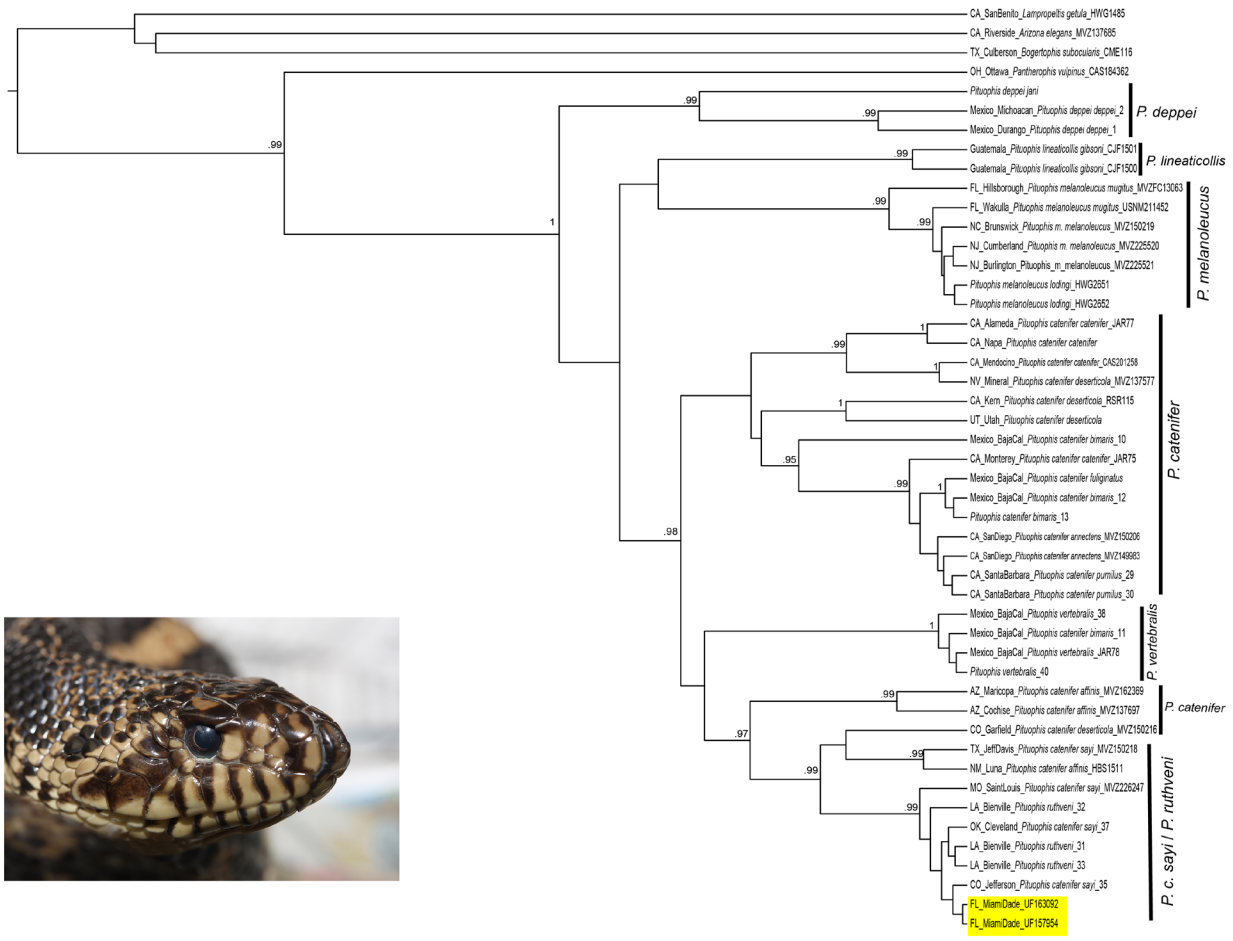

Figure 4. Bayesian Inference phylogeny for *Pituophis* (Squamata: Colubridae) snakes, including the two known *P. ruthveni* (highlighted in yellow, UF-Herpetology 157954 and 163092) collected in Miami, Miami-Dade County, Florida. Note that values (≥ 95%) above nodes represent posterior probabilities. *Inset photograph of UF-Herpetology 163092 by Dustin C. Smith.*

phylogenetics method was used without relying on a potentially arbitrary molecular clock (Zuckerkandl and Pauling 1965) that might incorporate uncertainty in the tree estimation process (Drummond et al. 2006). An uncorrelated lognormal relaxed clock with coalescent constant population size (Kingman 1982), estimated base frequencies, randomly generated starting tree, and normal distribution for the ucld.mean parameter priors were used. Two independent runs were performed consisting of three heated and one cold Markov Chain Monte Carlo (MCMC) estimated for 40 million generations, with every 1,000th sample being retained. Both MCMC runs were analyzed independently (to confirm chains were converging and not sampling local optima) using Tracer (ver. 1.6) for ESS values >200, as well as for a split standard deviation less than 0.005 for -lnL tree values among chains that indicate parameter stationarity was achieved. Trees sampled prior to stationarity were discarded as burn-in, which occurred prior to five million generations. Trees from both independent MCMC runs were combined and burn-in was removed using LogCombiner (ver. 1.8), the best statistically supported tree (i.e., Maximum clade credibility tree) with mean heights was obtained using TreeAnnotator (ver. 1.8), and a phylogenetic hypothesis with posterior probabilities was created using FigTree (ver. 1.4).

The most credible inferences of phylogenetic relationships were confined to nodes where nonparametric bootstrap values ≥ 70% and posterior probability (Pp) was ≥ 95% (Hillis and Bull 1993; Felsenstein 2004).

Morphology and color pattern

We determined sex, snout-vent length (SVL), tail length, number of ventrals, subcaudals, supralabials, infralabials, preoculars, postoculars, temporals, loreals, and dorsal scale rows; and color pattern of dorsum and venter. We compared these data to those found in the literature.

Results

Phylogenetic analyses.—Both ML and BI methods produced identical phylogenetic groupings (Figs. 3 and 4). Although some of these clades are organized differently in relation to one another the monophyly of *Pituophis* is well supported, which is congruent with the findings by Pyron and Burbrink (2009), though the latter study used only single samples for each species. Both of

our two *Pituophis* specimens have the same mtDNA haplotype, and both phylogenetic methods place them within the *P. catenifer sayi* / *P. ruthveni* clade.

Morphological data for UF-Herpetology 157954 include 226 ventrals, 55 subcaudals, 8/8 (left/right) supralabials, 11/11 infralabials, 1/1 preoculars, 7/7 postoculars, 4 temporals, 1/1 loreals, 27–30–24 dorsal scale rows, 34 body blotches, 8 tail blotches, parietal stripe present, and heavily patterned venter. Data for UF-Herpetology 163092 include 212 ventrals, 57 subcaudals, 8/8 (left/right) supralabials, 11/11 infralabials, 1/1 preoculars, 7/7 postoculars, 4 temporals, 1/1 loreals, 27–31–23 dorsal scale rows, 32 body blotches, and 11 tail blotches.

Discussion

Our ML and BI phylogenies produced identical main phylogenetic groupings (Figs. 3 and 4) as those found in the ML analysis by Rodriguez-Robles and De Jesus-Escobar (2000). However, we found no support for some relationships, and no support values are provided on the original ML tree by Rodriguez-Robles and De Jesus-Escobar (2000). Our two *Pituophis* specimens were placed within a well-supported *P. catenifer sayi* / *P. ruthveni* clade, the same group of specimens (except for our Florida specimens) uncovered by Rodriguez-Robles and De Jesus-Escobar (2000). *Pituophis catenifer sayi* and *P. ruthveni* were also found to be sister taxa based on a combined mtDNA and single nuclear (nDNA) (Pyron and Burbrink 2009) and phenetic morphological similarity (Reichling 1995) analyses. Nonetheless, we found no molecular support for the recognition of *P. ruthveni* as a separate species. One of the limitations of our and Rodriguez-Robles and De Jesus-Escobar's (2000) molecular studies is the use of only a single locus (ND4 region), and adding additional mtDNA and unlinked nDNA genes might provide needed data for distinguishing between these two named taxa. *Pituophis ruthveni* is currently recognized as a separate species because it occurs in allopatric populations and is believed to be diagnosable using color pattern characters, the most diagnostic being 28-38 dark dorsal body blotches and the blotches obscuring the ground coloration anteriorly (Collins 1991; Reichling 1995; Rodriguez-Robles and De Jesus-Escobar 2000). Our two *Pituophis* specimens exhibit these three characters, thus we categorized them as *P. ruthveni*.

Before our specimens were found, *Pituophis ruthveni* was not known to be kept at Zoo Miami, therefore this species is not representative of a zoo-mediated introduction pathway and was likely released by an outside person. Other species such as the Reticulated python, *Malayopython reticulatus* (see Kaiser et al. 2013; Reynolds et al. 2014), and Pacific Coast giant musk turtle, *Staurotypus salvinii*, are other examples of reptile species that have been illegally released on zoo property, the latter possibly established (Smith et al. 2011). Although we are currently uncertain if *P. ruthveni* is established in the vast protected undeveloped habitats surrounding public access areas, an adult male and gravid female were found suggesting reproduction might have taken place in the wild.

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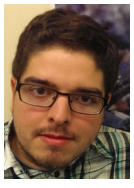
Dustin C. Smith is a conservation and research specialist at Zoo Miami. He graduated with a B.A. in 2001 from the University of South Florida. He has been involved with research projects in Costa Rica, Panama, and throughout the southeastern US, and is currently working on projects in Puerto Rico and Belize. Most of his current research involves native and non-native amphibians and reptiles in southern Florida.

Michael R. Rochford is the Invasive Species Coordinator at the University of Florida's Fort Lauderdale Research and Education Center, where he has worked for the last eight years. He received his B.S. in Biology from Kansas State University in 2004. He has worked extensively on radio-telemetry and diet studies of Burmese pythons in Florida as well as on projects to assess populations of American alligators and American crocodiles. In addition to his research and conservation interests, he also has strong interest in finding and photographing herpetofauna of the United States and Mexico.

Trey "Guy" Kieckhefer graduated with a B.S. in Biology from Eckerd College in Saint Petersburg, Florida. He worked for the University of Florida studying invasive Burmese pythons in the Everglades, and currently works at the Tavernier Science Center in the Florida Keys for the National Audubon Society. He conducts surveys for prey fishes in the Everglades and submerged aquatic vegetation. He is interested in Ichthyology, Ornithology, exotic species, and the overall health of the Everglades ecosystem.



Leroy P. Nuñez is a collections assistant within the Division of Herpetology at the Florida Museum of Natural History, University of Florida (UF). He obtained an Associate's Degree in Zoology from Santa Fe College and a Bachelor's Degree in Biology from UF. He is currently working on his Master's Degree in Interdisciplinary Ecology at the School of Natural Resources and Environment at UF. His current research involves studying phylogenies of invasive squamates in Florida and his future research projects will focus on phylogenetics and developmental evolutionary biology.