

# GENETIC CONFIRMATION OF TWO NONNATIVE SPECIES OF BUTTERFLY LIZARDS (LEIOLEPIDAE; *LEIOLEPIS*) WITH ESTABLISHED POPULATIONS IN FLORIDA, USA

Nicole R. Cobb<sup>1</sup>, Kenneth L. Krysko<sup>1</sup>, and Jan-Michael Archer<sup>1</sup>

## ABSTRACT

We used phylogenetic analysis to confirm the taxonomic identity of two nonnative butterfly lizard species (Agamidae; *Leiolepis belliana* and *L. rubritaeniata*) found in three southern Florida counties previously identified using only phenotypic characters. Our findings indicate that the Miami-Dade County population (*L. belliana*) is most genetically similar to a sample specimen from Chonburi, Thailand, whereas specimens from Charlotte and Lee Counties (*L. rubritaeniata*) are mostly closely related to sample specimens from Bangkok, Thailand. Our findings also support previous invasion hypotheses that butterfly lizard populations in Miami-Dade County stem from an introduction separate from the populations of Charlotte and Lee counties. More research is needed to determine the level of harm butterfly lizards pose to Florida's native species, but competition with native lizards for resources is suspected.

**Key words:** *Leiolepis*, butterfly lizard, Agamidae, PCR, invasion ecology, Florida.

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<sup>1</sup>Florida Museum of Natural History, University of Florida, Gainesville, FL 32611-7800 USA <cococobb@ufl.edu; kenneyk@ufl.edu; jjarcher@ufl.edu>

## INTRODUCTION

Traditionally, identification of a native species has been determined by direct observation focusing on an individual's coloration, locality (based on its known geographic distribution), and even scale morphology when trying to distinguish between closely related species that occur in sympatry. A problem arises, however, when identifying introduced species observed and collected outside of their indigenous ranges. These individuals can be misidentified due to potential similarities with the local native species, as well as researchers' unfamiliarity with foreign traits (Krysko et al., 2011a, 2011b). This is especially true when dealing with cryptic nonnative species, and even more so when only photographic vouchers are provided. More recently, identification of nonnative species has relied on using molecular techniques to compare genetic data between unknown introduced individuals and specimens collected in their native ranges.

Florida is a hot spot for introduced species, particularly reptiles, with the largest number of introduced amphibians and reptiles in the world (Krysko et al., 2011a; 2016). Lizards are an extreme example, with 16 native species compared to the 48 established nonnative species found in the state. Two of these are the diurnal Butterfly Lizard, *Leiolepis belliana* (Hardwicke and Gray 1827), and Red-banded Butterfly Lizard, *L. rubritaeniata* Mertens 1961 (Krysko et al., 2011, 2013).

*Leiolepis belliana* is native to Thailand, Myanmar, the Malay Peninsula, Pinang Island, Bangka, and Sumatra (Boulenger, 1903; De Rooij, 1915; Smith, 1935; Taylor, 1963; Rogner, 1997; Cox et al., 1999). Two subspecies are recognized: *L. b. ocellata* from Myanmar and northwestern Thailand, and *L. b. belliana* from all other areas noted (Rogner, 1997). Along with identifications based on photographs sent to another researcher, Krysko and Enge (2005) concluded that populations in Miami-Dade County correspond to the phenotype of *L. b. belliana*. Adults are brownish with yellow dorsal ocelli, flanks with bright orange and black transverse bars, and yellow dorsal striping is characteristic of neonates (Rogner, 1997). Krysko

and Enge (2005) also hypothesized that these populations originated from a pet dealer prior to 1992.

The Red-banded Butterfly Lizard, *Leiolepis rubritaeniata*, is indigenous to eastern Thailand, adjacent central and southern Laos, and southern central Vietnam (Peters, 1971; Stuart, 1999; Darevsky and Nguyen, 2004; Hartmann et al., 2012). Males can be identified by a highly reduced dorsal pattern (Grismer et al., 2008; Hartmann et al., 2012). The expandable lateral markings occur on the proximate post-axillary region; the posterior lateral two-thirds of the flanks are plain reddish-orange up to the groin; a lesser degree of dark lateral barring; and the edges of dorsal spots are fused in some places except for light yellowish post-and subocular stripes. Little to no color pattern exists on the side of the head, especially in older lizards, and only a polygonal net-like pattern made of the borders of faded ocelli are present (Grismer et al., 2008; Hartmann et al., 2012). Based on identifications from photographs sent to the same researcher above, Krysko et al. (2013) hypothesized that populations in Charlotte and Lee counties correspond to the phenotype of *L. rubritaeniata*. The invasion pathway for this species is not certain, but most likely via the pet trade (Krysko et al., 2013). Because these taxonomic determinations were originally made based solely on color patterns shown in photographs sent between U.S. researchers and a *Leiolepis* specialist in Europe, we herein use genetic analysis to test the putative species identities.

## MATERIAL AND METHODS

### LABORATORY TECHNIQUES

We obtained tissue samples from introduced Florida populations of suspected *Leiolepis belliana* and samples of *L. rubritaeniata* from museum specimens curated in the Division of Herpetology, Florida Museum of Natural History, University of Florida (UF-Herpetology) (Table 1). DNA was extracted using ZR Genomic DNA<sup>TM</sup>-Tissue MicroPrep (Zymo Research, LLC). Using total cellular DNA as a template and polymerase chain reaction (PCR) methodology (Saiki et al., 1988),

**Table 1.** Species, voucher number, locality, and GenBank accession number for Butterfly Lizards (genus *Leiolepis*) and outgroup (genus *Agama*) used in molecular analyses.

Species	Voucher	Locality	GenBank number	Source
<i>Leiolepis belliana</i>	UF 141589	USA, Florida, Miami Dade Co.		<i>This study</i>
<i>Leiolepis belliana</i>	UF 141590	USA, Florida, Miami Dade Co.		<i>This study</i>
<i>Leiolepis belliana</i>	UF 141591	USA, Florida, Miami Dade Co.		<i>This study</i>
<i>Leiolepis belliana</i>		Thailand, Chonburi	AB537554.1	Unpublished
<i>Leiolepis rubritaeniata</i>	UF 173364	USA, Florida, Charlotte Co.		<i>This study</i>
<i>Leiolepis rubritaeniata</i>	UF 167804	USA, Florida, Lee Co.		<i>This study</i>
<i>Leiolepis rubritaeniata</i>		Thailand, Nakonrachasima	AB480293.1	Unpublished
<i>Leiolepis rubritaeniata</i>		Thailand, Nakonrachasima	AB537553.1	Unpublished
<i>Leiolepis guentherpetersi</i>			AF378378.1	Unpublished
<i>Leiolepis guttata</i>			AF378377.1	Unpublished
<i>Agama picticauda</i>			GU128443	Unpublished

**Table 2.** Primers used to sequence the 16s region in Butterfly Lizards (genus *Leiolepis*).

Gene Region	DNA Marker	Primer Name	Primer sequence	Source
16s	mtDNA	A2290	CGC-CTG-TTT-ACC-AAA-AAC-AT	Kocher et al. 1989
16s	mtDNA	B2860	CCG-GTC-TGA-ACT-CAG-ATC-ACG-T	Kocher et al. 1989
16s	mtDNA	L52	CGT-GCA-AAG-GTA-GCA-CAA-TC	<i>This study</i>
16s	mtDNA	H455	CGG-ACC-CTT-GAT-AGC-TTC-TG	<i>This study</i>

mitochondrial DNA (mtDNA) was amplified and sequenced for the 16s region using primers A2290 and B2850 (Kocher et al. 1989), and L52 and H455 (this study; Table 2). PCR was conducted in 25 µl reactions: 9.5 µl H<sub>2</sub>O, 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 3 min, followed by 35 cycles of amplification: denaturing at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 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 trace files from the automated sequencer (Genomics Division, Interdisciplinary Center for Biotechnology

Research, University of Florida) were assembled and edited as necessary using Geneious software (ver. 6.1, created by Biomatters, available from <http://www.geneious.com>).

#### PHYLOGENETIC ANALYSES

We downloaded comparison DNA sequence data for 16S from GenBank for six samples, including *Leiolepis belliana*, *L. rubritaeniata*, *L. guentherpetersi*, *L. guttata*, and the outgroup taxon *Agama picticauda* (Table 1). All sequences were aligned with the Clustal algorithm using MEGA software (ver. 6, Tamura et al., 2013) and edited manually using SeaView (ver. 4.2.5, Gouy et al., 2010) when necessary.

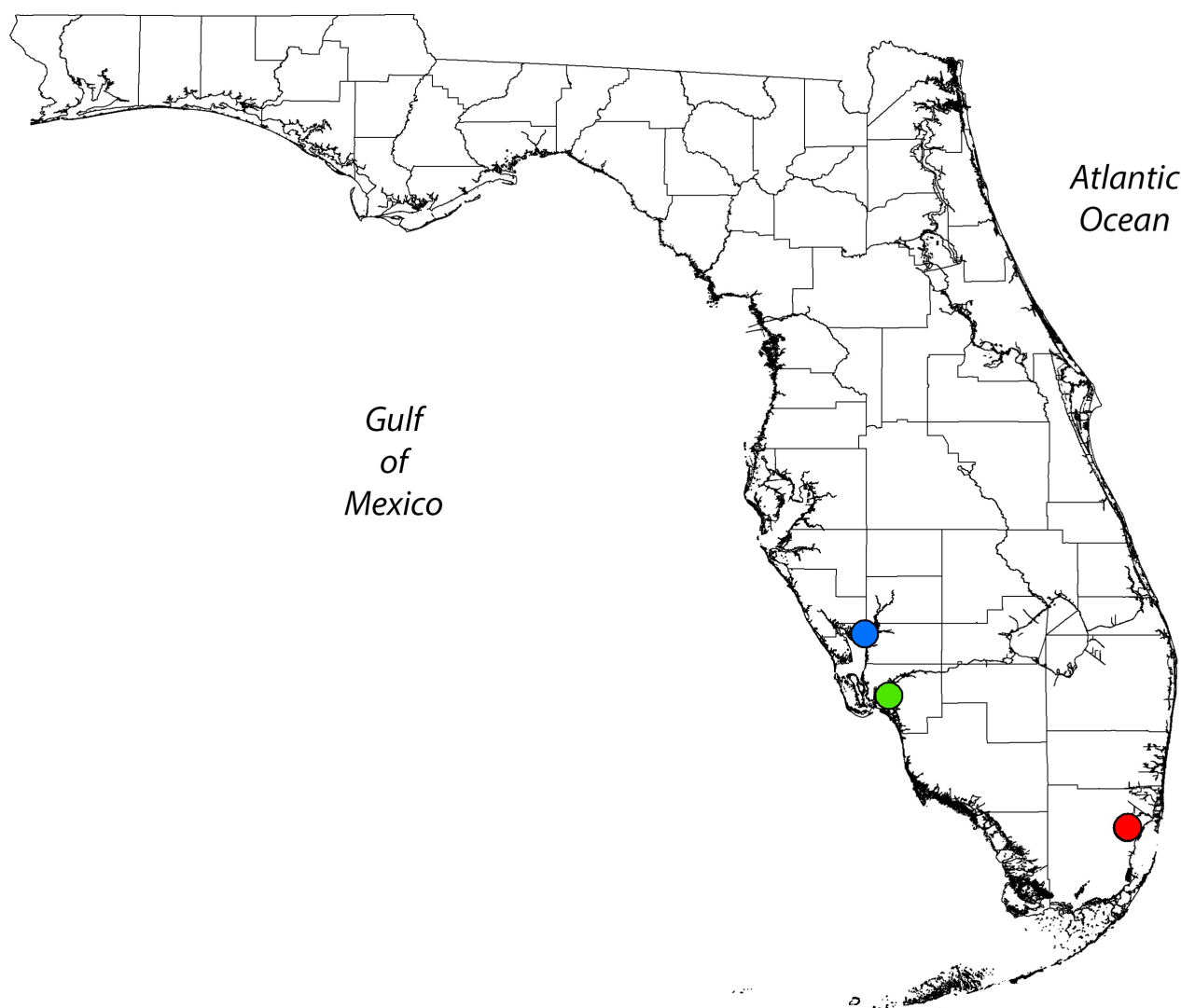
We obtained unique haplotypes using DnaSP (ver. 5.10.01; Rozas, 2009). Relationships among haplotypes were estimated using Maximum Likelihood (ML) methodology with the Tamura-Nei

model, complete deletion mechanism, nucleotide substitution, nearest-neighbor interchange heuristic method, very strong branch-swap filter, and 1,000 nonparametric bootstrap replicates (Felsenstein, 1985) to assess node support using MEGA (Tamura et al., 2013). The most credible support of phylogenetic relationships was confined to nodes where nonparametric bootstrap values were  $\geq 70\%$  (Hillis and Bull, 1993; Felsenstein, 2004).

### RESULTS

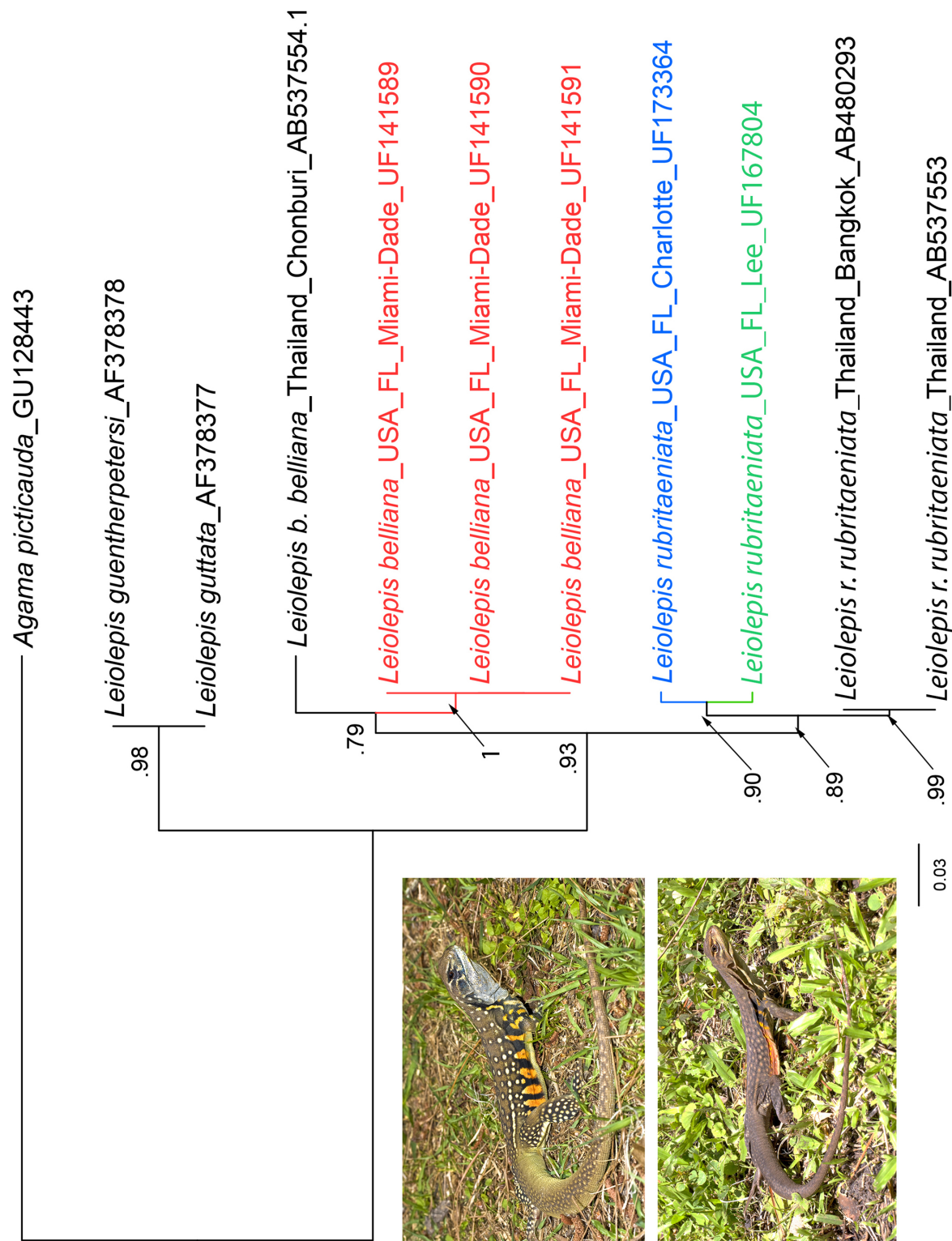
We analyzed 501 base pairs (bp) of sequence data.

We found seven haplotypes in our data set, three of which are *Leiolepis* from Florida. One haplotype was found in each of Charlotte, Lee, and Miami-Dade counties (Fig. 1). Our genetic data confirm the introduction and species identity of both *Leiolepis belliana* and *L. rubritaeniata*. *Leiolepis belliana* from Miami-Dade County are most closely related to an individual from Chonburi, Thailand, whereas *L. rubritaeniata* from Charlotte and Lee counties are most closely related to individuals from Bangkok, Thailand (Fig. 2).



**Figure 1.** Map of the state of Florida showing the distribution of *Leiolepis* based on vouchered records from the Florida Museum of Natural History. Red circle indicates individuals with haplotype A, blue circle indicates haplotype B, and green circle indicates haplotype C.





**Figure 2.** Maximum-likelihood phylogeny of *Leiolepis* and outgroup taxon *Agama picticauda*. Note that values above major nodes represent bootstrapped support  $\geq 70\%$ , and samples highlighted in red, blue, and green represent different haplotypes for specimens collected from introduced populations in Florida. Inset photographs include: *L. belliana* (UF-Herp 144200; top) and *L. rubritaeniata* (UF-Herp 173364; bottom).

## DISCUSSION

One haplotype found among samples of *Leiolepis belliana* from Miami-Dade County populations suggested they originated from a single source and support the invasion hypothesis (Krysko and Enge, 2005). One haplotype found in each of the populations from Charlotte and Lee counties suggested they were independent introductions. Larger sample sizes from throughout the native ranges of both species would help confirm the species origins we documented with genetic analysis.

We do not know if these two species of butterfly lizards are harmful to native Florida lizards. Both eat vegetation, crabs, grasshoppers, beetles, larval butterflies, and other insects (Krysko and Enge, 2005). This suggests they could compete with Florida's native lizard species (i.e., anoles, racerunners, skinks, etc.) that share the same type of diet should resources become scarce. Because they have access to a wider range of resources, their broad diet might also give them an advantage over native lizards that are more specialized consumers. If introduced populations of butterfly lizards are determined to have a competitive advantage, then they should be added to the list of invasive species in Florida. Invasive species are a major threat to the stability of native ecosystems (Reed and Krysko, 2014). Continued use of molecular techniques is necessary to assess correctly the number of nonnative and invasive species. Ecological experiments could then assess potential threats such as competition for food resources.

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