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Evidence of Population Genetic Structure within the Florida Worm Lizard, *Rhineura floridana* (Amphisbaenia: Rhineuridae)

ABIGAIL MULVANEY,[1] TODD A. CASTOE,[1] KYLE G. ASHTON,[2] KENNETH L. KRYSKO,[3] AND CHRISTOPHER L. PARKINSON[1,4]

[1]*Department of Biology, University of Central Florida, 4000 Central Florida Boulevard, Orlando, Florida 32816-2368, USA*
[2]*Department of Biology, Kutztown University, Kutztown, Pennsylvania 19530, USA*
[3]*Florida Museum of Natural History, Division of Herpetology, University of Florida, Gainesville, Florida 32611, USA*

ABSTRACT.—**The Florida Worm Lizard (*Rhineura floridana*) is the only extant representative of the suborder Amphisbaenia occurring in the United States and the only living representative of the Rhineuridae. We updated the known distribution of this species from 510 records with known localities. We further examined geographic genetic structure within this species using 1360 bp of mitochondrial DNA sequence data from 18 samples of *R. floridana*. Our results suggest an ancient divergence between populations in the north-central Florida peninsula from populations in the south-central peninsula. High genetic distances are observed within south-central populations, whereas genetic structure within northern populations is less discrete and characterized by much shallower divergences. Our findings suggest that south-central populations may be candidates for taxonomic recognition (or recognition as distinct management units) if additional genetic and morphological data support our results.**

The Florida Worm Lizard, *Rhineura floridana* (Baird, 1858), is the only extant representative of the suborder Amphisbaenia occurring in the United States and the only living representative of the Rhineuridae (Kearney, 2003). Like other amphisbaenians, this species exhibits extreme adaptations to a fossorial lifestyle, including a shovel-like head with a countersunk lower jaw and lack of exterior eyes, ear openings, and limbs. As a result of its fossorial habits, it is uncommonly observed, and many facets of its natural history, distribution, relative abundance, and geographic variation are poorly known.

Higher-level relationships among amphisbaenians have remained unresolved for more than a century. Recent studies (i.e., Kearney, 2003; Macey et al., 2004) have suggested a distant relationship between *R. floridana* and other extant amphisbaenians, highlighting an extensive hiatus in distribution between *R. floridana* and its closest extant relatives. In contrast to the present-day restricted and isolated range of *R. floridana*, a rich fossil record indicates that rhineurids once occurred across North America, although rhineurid fossils postdating the Miocene have been found exclusively in Florida (Zug, 1968; Holman, 1995). Collectively, fossils of *R. floridana* illustrate the presence of this species in Florida through the Pleistocene (Holman, 1958, 1959, 1962, 1995, 1996). Today, *R. floridana* occurs in a wide range of habitats, from dry upland scrub to lower-lying hammocks, throughout northern and central peninsular Florida and a single locality in Georgia.

Zug (1970) hypothesized that *R. floridana* colonized peninsular Florida by the end of the Pliocene (approximately 1.8 mya) and was subsequently subdivided by the Aftonian inundation (early Pleistocene; Cooke, 1945; MacNeil, 1950) into an island population associated with the Lake Wales Ridge, and a mainland population in the northern Florida peninsula. Populations from the Lake Wales Ridge differ in several morphological characters from more northern populations (Zug, 1968), potentially supporting this assertion. Yet, Zug (1970) identified a zone of apparent intergradation between Lake Wales Ridge populations and populations centered in Polk County near the northern extent of the Lake Wales Ridge. This apparent zone of intergradation led Zug (1968, 1970) to presume ongoing gene flow between northern and southern forms and, for this reason, he did not taxonomically subdivide *Rhineura*.

In this study, we evaluate population structure within *R. floridana* by combining updated distribution data with geographic patterns of mtDNA genetic structure. We compare our resolution of population structure of *R. floridana* with data from previous studies of morphological variation within *R. floridana* and population structure of codistributed lizards. We discuss taxonomic and

[4] Corresponding Author. E-mail: cparkins@pegasus.cc.ucf.edu

TABLE 1. Voucher, locality, and GenBank accession numbers for *Rhineura floridana* samples used in molecular analyses. Abbreviations for voucher specimens follow Leviton et al. (1985) except: CLP = Christopher L. Parkinson, PEM = Paul E. Moler.

Specimen ID	Voucher	Locality	GenBank Accession	
			ND4	12S
R.f.-Alachua - #1	UF 121175	Alachua County 29.6164 N, 82.3361 W	AY881076	AY881093
R.f.-Alachua - #2	UF 112550	Alachua County 29.6549 N, 82.3173 W	AY881074	AY881091
R.f.-Alachua - #3	UF 121174	Alachua County 29.6164 N, 82.3361 W	AY881075	AY881092
R.f.-Alachua - #4	UF 123332	Alachua County 29.6342 N, 82.3753 W	AY881077	AY881094
R.f.-Alachua - #5	UF 132744	Alachua County 29.5951 N, 82.3404 W	AY881079	AY881096
R.f.-Alachua - #6	CLP 291	Alachua County 29.5016 N, 82.4266 W	AY881071	AY881088
R.f.-Alachua - #7	CLP 292	Alachua County 29.50166 N, 82.4266 W	AY881072	AY881089
R.f.-Citrus - #8	CAS 195955	Citrus County 28.7502 N, 82.2967 W	AY881065	AY881081
R.f.-Citrus - #9	CAS 214845	Citrus County 28.6899 N, 82.3380 W	AY881067	AY881083
R.f.-Citrus - #10	CAS 200844	Citrus County 28.8359 N, 82.3303 W	AY881066	AY881082
R.f.-Citrus - #11	PEM 1	Citrus County 28.7883 N, 82.3217 W	—	AY881098
R.f.-Citrus - #12	PEM 2	Citrus County 28.8550 N, 82.3330 W	AY881080	AY881099
R.f.-Highlands - #13	CLP 201	Highlands County 27.4971 N, 81.4413 W	AY881068	AY881084
R.f.-Highlands - #14	CLP 202	Highlands County 27.2925 N, 81.3800 W	AY881069	AY881085
R.f.-Manatee - #15	UF 128407	Manatee County 27.6717 N, 82.3527 W	AY881078	AY881095
R.f.-Orange - #16	CLP 293	Orange County 28.7067 N, 81.5400 W	AY881073	AY881090
R.f.-Seminole - #17	CLP 203	Seminole County 28.7118 N, 81.4540 W	AY881070	AY881086
R.f.-Sumter - #18	UF 137171	Sumter County 28.5875 N, 82.2305 W	—	AY881097

conservation implications of distinct evolutionary lineages identified within *R. floridana* and highlight important unresolved questions that need to be addressed in future studies.

MATERIALS AND METHODS

Geographic Range Compilation.—We obtained locality records of *R. floridana* from the literature (Gans, 1967; Zug, 1968, 1970; Meylan, 1984; Hingtgen, 1991; Wood, 1995; Jensen and Payne, 1996) and from systematic collections throughout the United States. Following Zug (1968), we did not include unsubstantiated reports of specimens further south than Highlands County (e.g., Telford, 1959). We also did not include questionable records of one specimen from Georgia and another from Liberty County, Florida (see Zug, 1968; Meylan, 1984; Wood, 1995). For specimens with unknown geographic coordinates, we estimated latitude and longitude based on the locality data for each specimen. Localities were plotted using ArcView v3.0 (ESRI).

Laboratory Methods.—Mitochondrial gene sequences were analyzed for 18 samples of *R. floridana* collected from seven Florida counties (Table 1). Genomic DNA was isolated from tissue and skeletal samples by standard proteinase-K digestion. This was followed by purification using the DNeasy extraction kit and protocol (Qiagen), or standard phenol/chloroform methods using the Phase Lock Gel kit and protocol (Eppendorf).

Two mitochondrial gene regions were amplified, sequenced, and analyzed: a portion of the protein-coding NADH dehydrogenase subunit 4 gene and three downstream tRNAs (approximately 900 total bp; called ND4 hereafter), and a portion of the 12S small subunit rRNA gene (approximately 500 bp; called 12S hereafter). The

ND4 region was amplified using the primers (ND4 and LEU) and amplification conditions described in Arévalo et al. (1994). The 12S fragment was amplified using the primers L1091F (Knight and Mindell, 1993) and 12e (Wiens et al., 1999) and conditions described in Parkinson (1999). Details of PCR chemistry or thermal cycling parameters are available upon request. Positive PCR products were excised from agarose electrophoretic gels and purified using the GeneCleanIII kit (BIO101) or MiniElute Gel Extraction Kit (Qiagen). Purified PCR products were sequenced in both directions with the amplification primers. An additional internal primer (HIS; Arévalo et al., 1994) was used to sequence the ND4 fragment. Samples that could not be sufficiently sequenced directly were cloned using the Topo TA 2.1 cloning kit (Invitrogen). Plasmids were isolated from multiple clones per individual using the Qiaquick spin miniprep kit (Qiagen). Plasmids were sequenced using M13 primers (provided by Topo TA kit, Invitrogen). Purified PCR products and plasmids were sequenced using the CEQ D Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter) and run on a Beckman CEQ2000 automated sequencer.

Raw sequence chromatographs were edited using Sequencher v4.1 (Gene Codes Corp.). In cases where gene fragments were cloned, chromatographs from multiple clones, as well as partial sequences from directly sequenced PCR products, were combined and edited together for each specimen. These sequences were later rechecked for positive alignment based on amino acid sequence (protein-coding ND4 region). Alignment was unambiguous, and no indels or stop codons were found in protein-coding regions of ND4. All sequences were deposited in GenBank (Table 1).

Analyses of Molecular Data.—We conducted separate and combined analyses for the two mtDNA sequences. All mitochondrial genes are inherited as a single linkage unit, thus we expected that phylogenies from different mitochondrial genes would estimate convergent gene trees. We used the incongruence length difference (ILD) test (Farris et al., 1994; implemented in PAUP* with 100 branch-and-bound replicates) to test for disagreement in phylogenetic signal between independent gene datasets. We estimated nucleotide diversity (π; Nei, 1987) and pairwise genetic distance measures using DnaSP v4.0 (Rozas et al., 2003), Mega2 (Kumar et al., 2001), and PAUP* v4.0b10 (D. L. Swofford, Sinauer Associates, Sunderland, MA 2002). To accommodate two individuals that had only one of two genes sequenced (Table 1), all diversity and distance measures were conducted with missing sites excluded only for necessary pairwise comparisons.

Phylogenetic analyses were conducted using PAUP* v4.0b10 (D. L. Swofford, Sinauer Associates, Sunderland, MA 2002). Phylogenetic reconstruction was accomplished using both maximum parsimony (MP) and maximum likelihood (ML) methods. Given the lack of an appropriate extant outgroup, all reconstructions were not rooted a priori but were later midpoint rooted for presentation. For the MP analyses, we used equally weighted parsimony and searched for optimal trees with the branch-and-bound algorithm. Gaps in alignment were treated as missing data. Support for nodes was assessed by conducting 1000 nonparametric branch-and-bound bootstrap pseudoreplicates (Felsenstein, 1985). We used ModelTest v3.06 (Posada and Crandall, 1998) to determine the simplest best-fit model of evolution for use in ML analyses (based on successive hierarchical log-likelihood ratio tests). Using the selected model and starting parameters, we heuristically searched for optimal ML trees with PAUP*. Heuristic ML searches were conducted with 100 random taxon addition sequences. Nodal support for ML trees was assessed with 100 full-heuristic bootstrap pseudoreplicates (each with 10 random taxon addition sequence replicates).

Results

Geographic Range.—Our records for *R. floridana* ($N = 510$) illustrate that this species occurs in a total of 28 Florida counties from Highlands County in the south-central Florida peninsula north to Lanier County, Georgia (Fig. 1).

Results of Molecular Analyses.—Of the 1360 total characters, the ND4 dataset provided 891 characters, 190 of which were variable and 85 of which were parsimony-informative. The 12S dataset provided 469 characters, 55 of which were variable and 36 of which were parsimony-informative. Using combined data, the maximum pairwise divergence between samples was 12.0%, and the mean pairwise divergence across all samples was 4.53%. Nucleotide diversity and between-group genetic distances are summarized in Table 2, and pairwise genetic distances between all samples are provided in Appendix 1.

Separate phylogenetic analyses of ND4 and 12S datasets (not shown) resulted in concordant estimates of relationships, differing only with respect to weakly supported nodes (bootstrap of conflicting nodes $< 60\%$). Results of the ILD test suggested that independent gene datasets produced essentially identical phylogenetic signal ($P = 1.0$), failing to reject the null hypothesis of incongruent trees from independent gene datasets, and further justifying the combining of datasets.

Based on hierarchical log-likelihood ratio tests conducted in ModelTest, the simplest best-fit

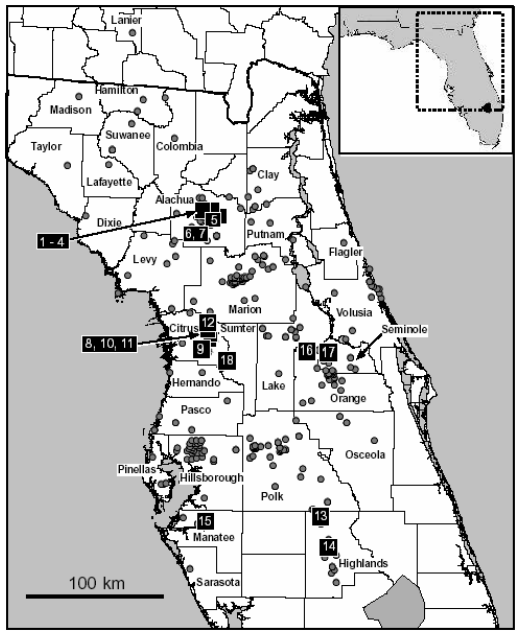

FIG. 1. Range map including all known collecting localities for *Rhineura floridana* (N = 510). County names are shown only for counties with recorded occurrences of *R. floridana*. Specimen localities are indicated by circles. Localities of individuals that were sampled for molecular data in this study are indicated by squares. Numbers appearing in squares correspond to sample numbers in Table 1.

model of nucleotide evolution was the K81+G with the following parameters: freq.A = 0.3391, freq.C = 0.2898, freq.G = 0.1460, freq.T = 0.2251, r[A–C] = r[G–T] = 1.0000, r[A–G] = r[C–T] = 6.9670, r[A–T] = r[C–G] = 0.1044, gamma = 0.3137. This model and starting parameters were used to initiate ML searches. The ML search resulted in two equally likely phylogenetic solutions (-lnL = 3440.47053). The strict consensus of these is provided (Fig. 2A). These two topologies differed only in the resolution of a clade including samples "*R.f.*-Citrus-#8" and "*R.f.*-Sumter-#18" (collapsed in Fig. 2A). The MP search resulted in 15 equally parsimonious trees (303 steps, homoplasy index = 0.116, rescaled consistency index = 0.894). The majority-rule consensus of these is presented (Fig. 2B). MP and ML methods reconstructed essentially the same tree topologies, differing only with respect to resolution of terminal nodes.

Genetic results show a deep divergence between samples from the south-central peninsula in Manatee and Highlands counties from those in the north-central and northern peninsula (Fig. 2A, B). The mean genetic distance between haplotypes in these two clades was 9.27% (Table 2). All three individuals within the south-central clade (Fig. 2A, B) possess substantially divergent haplotypes (within clade π = 7.34%; Table 2). The north-central/northern clade (Fig. 2A, B) contained the majority of haplotypes sampled (N = 15) and is divided into two subclades that geographically overlap in Alachua County; a northern peninsula-Alachua clade comprising exclusively Alachua County samples (within clade π = 0.269%), and a north-central/northern clade including samples from Alachua, Citrus, Orange, Seminole, and Sumter Counties (within clade π = 1.23%; Table 2). The north-central/northern clade (mean 2.44% divergence from the northern peninsula-Alachua clade; Table 2) includes a divergent group of haplotypes from Seminole and Orange counties in eastern-central Florida, sister to a clade of haplotypes from more northern and western areas in Alachua, Citrus, and Sumter Counties.

TABLE 2. Nucleotide diversity statistics for sampled populations of *Rhineura floridana*. Clade names in the left column correspond to Figure 2.

	Sample size	Mean distance between groups	Nucleotide diversity
North-central/northern clade	15		1.72%
Northern-Alachua clade vs. Northern-central clade	5 vs. 10	2.44%	
Northern-Alachua clade	5		0.27%
Northern-central clade	10		1.23%
Alachua County	7		1.32%
Citrus County	5		0.59%
South-central clade	3		7.34%
Highlands County	2		5.24%
Highlands County vs. Manatee County	2 vs. 1	8.63%	
North-central/northern clade vs. South-central clade	15 vs. 3	9.27%	
All localities	18		4.05%

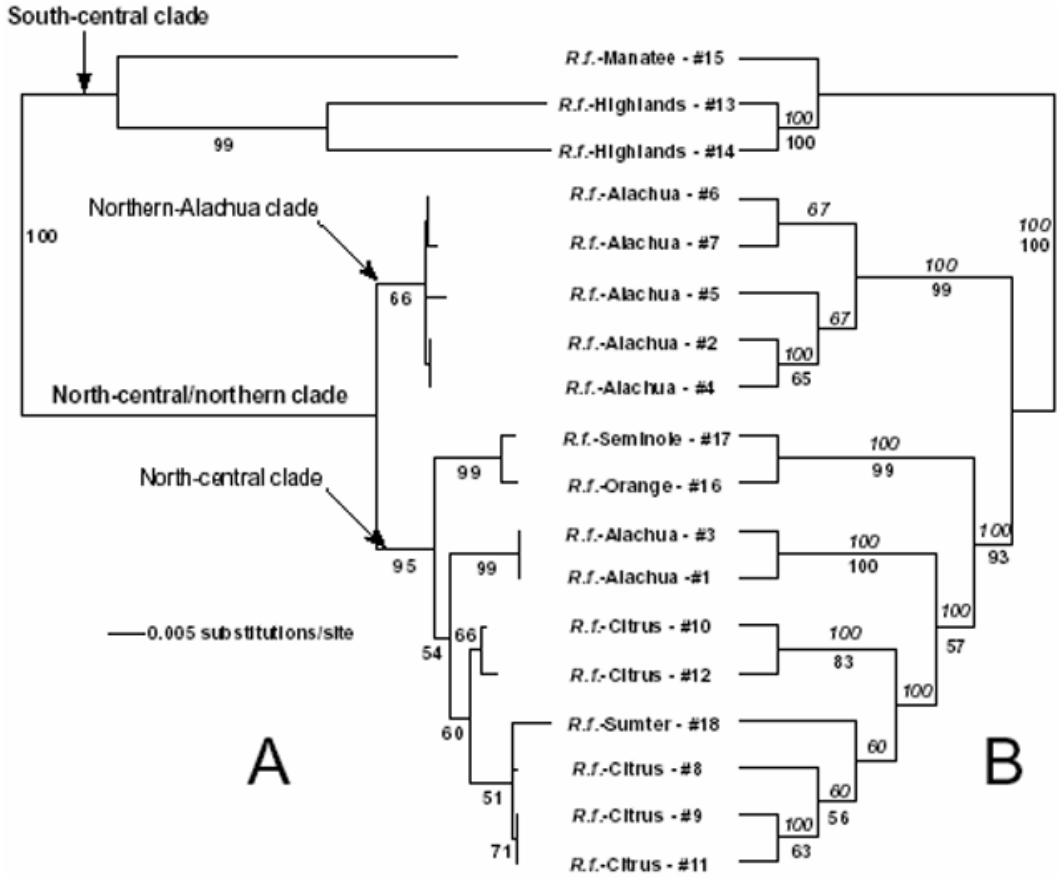

FIG. 2. Phylogenetic relationships among *Rhineura floridana* individuals sampled for molecular data. The naming scheme used for operational taxonomic units indicates the Florida county of each specimen and the sample number (see Table 1). Nodal support based on nonparametric bootstrap analyses are indicated as percentages (in **bold**) adjacent to corresponding nodes (values < 50% not shown). The tree on the left (A) is a strict consensus phylogram of two equally likely trees resulting from maximum likelihood analyses. Major clades are labeled to facilitate in-text discussion. The tree on the right (B) is a 50% majority-rule consensus cladogram (including other compatible groupings) of 15 equally parsimonious trees resulting from equally weighted maximum parsimony analysis. Numbers above internodes (in *italics*) represent the frequency of node resolution across the 15 equally parsimonious trees.

DISCUSSION

Distribution.—Our updated distribution map (Fig. 1) provides a substantial advancement in our understanding of the geographic continuity and isolation of *R. floridana* populations. From these data, it is apparent that *R. floridana* ranges throughout northern peninsular Florida, most of central peninsular Florida, with one record from southern Georgia and the most southern peninsular Florida records restricted to the Lake Wales Ridge and the west coast (Fig. 1).

Genetic Population Structure and Phylogeography.—Patterns of mtDNA haplotype diversity in *R. floridana* demonstrate substantial genetic structure and sequence divergence between major regions in peninsular Florida. Many of these patterns generally coincide with those identified by Branch et al. (2003) for *Eumeces egregius*, *Neoseps reynoldsi*, and *Sceloporus woodi* (also see Clark et al., 1999). Our results, and those of Branch et al. (2003), demonstrate deep mtDNA divergences between populations in south-central Florida (associated with the Lake Wales Ridge) and populations in the north-central and northern peninsula. Mean pairwise sequence divergence between individuals from our north-central/northern and south-central clades for *R. floridana* is 9.27%.

In addition to deep divergences between these major clades, we found substantial genetic divergence between south-central populations, both within Highlands County (5.24%), and

between Highlands and Manatee counties (mean 8.63%). The divergent samples from Highlands County are separated by Josephine Creek, suggested to be an apparent barrier to gene flow for *S. woodi* (Clark et al., 1999) and some insect species (Deyrup, 1996).

The sample sizes available for our study limit specific conclusions regarding population structure and gene flow among more northern populations. Although we found moderate sequence divergence between northern peninsular haplotypes (mean 2.44% divergence between the northern-Alachua clade vs. north-central/northern clade), samples do not form monophyletic groups based on geography (e.g., Alachua and Citrus County samples, Fig. 2). This may suggest that, historically, populations were more isolated in the north, promoting differentiation of haplotype lineages, followed by more recent gene flow among these populations acting to geographically mix haplotype subclades.

Regardless of potential biases introduced by limited population sampling, our results show haplotype divergences among south-central populations that are much higher than among more northerly populations. This suggests the influence of either older isolation events between south-central populations and/or higher levels of habitat fragmentation or connectivity through history. Zug (1968) suggested that *R. floridana* populations in south-central Florida demonstrated higher habitat specificity than those to the north, which is tentatively supported by individuals in the south being encountered almost exclusively in xeric habitats. Our results add to the literature demonstrating the taxonomic and genetic uniqueness of many flora and fauna inhabiting the central Florida ridges (e.g., Deyrup, 1989; McCoy and Mushinsky, 1992; McDonald and Hamrick, 1996; Branch et al., 2003) and corroborate evidence for substantial differentiation among populations within these xeric upland systems (Deyrup, 1996; McDonald and Hamrick, 1996; Branch et al., 2003).

Taxonomic Implications.—Overall patterns of genetic structure within *R. floridana* are broadly comparable to patterns based on morphological characters (Zug, 1968). Both molecular and morphological data illustrate strong differentiation of south-central populations from north-central/northern Florida populations that include the type locality (Micanopy, Alachua County; Baird, 1859). Zug (1968, 1970) provided morphological evidence for an intergradation zone between Lake Wales Ridge populations and more northerly populations in Polk County. Zug (1968) also suggested that populations in Manatee County represented an intermediate phenotype (along with populations from Polk County) between Lake Wales Ridge populations and populations from northern and north-central Florida. Our genetic results suggest that the Manatee County population may be a third divergent lineage of *Rhineura* distinct from Lake Wales Ridge populations and populations in the north-central and northern peninsula. We were unable to incorporate Polk County samples for molecular analyses; thus, our results are insufficient to resolve the degree of reciprocal genetic isolation among populations across this potential contact zone, or allow us to evaluate potential affinities between Polk and Manatee County specimens. This lack of resolution prevents us from recognizing what otherwise appear to be well-differentiated south-central populations as a distinct evolutionary species.

Despite the limitations of this study, our results provide a framework of testable hypotheses that require consideration (through increased geographical and molecular sampling) to resolve outstanding questions regarding the historical biogeography, population genetic structure, taxonomy, and conservation of *R. floridana*. Determination of the affinities of Polk County populations relative to both Highlands and Manatee County populations is a priority for future studies. Increased distribution information and genetic sampling across the southern portion of the range of *R. floridana* (including De Soto, Hardee, Highlands, Hillsborough, Manatee, and Polk counties) will be critical for the taxonomic recognition of apparently divergent evolutionary lineages within *R. floridana* and definition of units for conservation.

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