Journal of Herpetology, Vol. 55, No. 1, 62–69, 2021

A Case of Mistaken Identity: Genetic and Anatomical Evidence Reveals the Cryptic Invasion of *Xenopus tropicalis* in Central Florida

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ABSTRACT.—**Nonnative species are drivers of global change, affecting biodiversity and burdening society with economic costs. Effective management of an invasion relies on the ability to make accurate predictions about the target species' spread and impact. This requires knowledge of the target species' biology, making taxonomic validation critical. Even so, external morphology is still widely used to determine the species identity of novel invaders. Here, we show that a nonnative pipid frog population in Riverview, Florida, USA, initially identified as African Clawed Frog (*Xenopus laevis*), is a cryptic invasion of Tropical Clawed Frog (*Xenopus tropicalis*) and the only known nonnative population of this species. We used DNA sequence data and osteology from high-resolution microcomputed tomography to confirm this identification. Furthermore, we conducted field surveys to delineate the population's invaded range in Florida. We detected the presence of adult *X. tropicalis* at 22 sites and larvae at a subset of 12 sites, representing an occupied area of approximately 1,630 ha. Differing body size and physiology of these two species of *Xenopus* suggest considerable differences in their impact, spread, and potential geographic range.**

Nonnative species present a major hazard to global biodiversity (Mack et al., 2000) and are one of the largest drivers of contemporary extinction (Bellard et al., 2016). Furthermore, nonnative species threaten human and ecosystem health (Juliano and Lounibos, 2005; Pejchar and Mooney, 2009) and cost the United States alone approximately $120 billion annually (Pimentel et al., 2005). Given these large costs, prevention of establishment and containment of extant invasions are key to preserving biodiversity (Molnar et al., 2008). Containment can require intensive and long-term management, aimed at controlling the spread of the invader (Meyer et al., 2011). Effective management of nonnative species often requires an approach tailored to the target species' biology (Lennox et al., 2015), making it imperative to correctly identify the species of interest. The failure to correctly identify a nonnative species is a phenomenon known as cryptic invasion (Geller et al., 2010).

Researchers often prioritize morphological features to assign taxonomic status, and as a result, cryptic invasions abound (Morais and Reichard, 2018). Although external morphology can be reliably used to distinguish certain species, closely related species often require molecular approaches to disambiguate (Bruschi et al., 2013). Otherwise, cryptic invaders may go unnoticed (Verloove, 2010; Hill, 2017) and known invaders may continue to proliferate (Thum et al., 2012). Taxonomic confusion can also reduce the reliability of models aimed at quantifying the target invader's potential for range expansion (Mori et al., 2018). This is particularly true for ecological niche models (ENMs; Hernandez et al., 2006), which rely on environmental data collected from within the target species' native range (Peterson, 2003).

In 2016, Hill et al. (2017) discovered a high-density breeding population of an African Clawed Frog of the genus *Xenopus* in west central Florida. Initially identified as *Xenopus laevis*, subsequent inspection of adult individuals revealed external morphology more consistent with members of the subgenus *Silurana* (Evans et al., 2015). In addition, experimental evidence suggests that individuals within the west central Florida population may be more adapted to higher temperature regimes and reduced thermal variation. Specifically, individuals from within the west central Florida population are characterized by an increased heat tolerance, reduced cold tolerance, and more narrow thermal breadth, relative to *X. laevis* (Goodman et al., 2019; Araspin et al., 2020). Here, we resolve the taxonomic confusion concerning the Florida population by using analyses of DNA sequence data and osteological data from microcomputed tomographic (CT) scans. These analyses reveal this population to be the world's only known nonnative population of *X. tropicalis*, thereby providing a unique opportunity for making comparisons with the invasion biology of the well-studied and widely invasive *X. laevis*. In addition, we provide detailed information on the current distribution of *X. tropicalis* in Florida and discuss the implications for future management.

MATERIALS AND METHODS

DNA Sequencing.—We conducted all laboratory work at the Florida Museum of Natural History. We extracted genomic DNA from tissues (liver, muscle, or toe clips) by using Qiagen DNeasy kits, following their protocol for animals. Using polymerase chain reaction (PCR), we amplified an ~520-base pair fragment of mitochondrial DNA that encodes part of the mitochondrial ribosomal 16S gene (95°C 30 s, 54°C 30 s, 72°C 1 min) by using 35 cycles and the oligonucleotide primers 16sar-L 16sbr-H (Palumbi et al., 2002). We used ExoSAP-IT (Affymetrixs) to purify all amplified PCR products and then shipped this product for Sanger sequencing at Genewiz. We deposited all sequences in GenBank (Appendix 1).

Alignment and Phylogenetic Analyses.—We created contigs in Geneious v.9.0.5 (Biomatters; http://www.geneious.com),

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DOI: 10.1670/20-083

aligned consensus sequences using MAFFT v.7.309 (Katoh and Standley, 2013), and checked manually for gross errors such as sequences in the wrong strand orientation. We then combined our sequences with the most complete taxonomic sampling of *Xenopus* to date (Evans et al., 2015). We trimmed the resulting block of aligned sequences so that all individuals had complete data, resulting in a final alignment of 119 samples, 498 base pairs in length with 113 informative sites. Using ModelFinder (Kalyaanamoorthy et al., 2017), we found that the best model of nucleotide substitution was GTR+F+I+G4. We created a maximum likelihood tree by using IQtree (Nguyen et al., 2015) with ultrafast bootstrapping (1,000 replicates; Hoang et al., 2018). We calculated the average pairwise distance between our Florida samples ($N = 16$) and nonidentical sequences of *X. tropicalis* from GenBank ($N = 12$), by using MegaX v.10.1.7 (Kumar et al., 2018). We applied a bootstrap (500 replicates) to estimate variance and assigned uniform rates of substitution within and between the Florida and native samples. There were no ambiguous positions in our final dataset.

Micro-CT Scanning.—To confirm the subgeneric identity, we used high-resolution CT. We performed all scans on ethanol-preserved specimens, at the University of Florida's Nanoscale Research Facility, by using a Phoenix v|tome|x M (GE Measurement & Control Solutions, Boston, MA, USA). We customized voltage and current for each specimen to balance resolution and intensity contrast. We scanned the entirety of four specimens and the anterior half of one additional specimen at an increased resolution (35.06 and 15.44 μm, respectively). We used GE's reconstruction software datos|x to convert x-ray images into tomogram images. We then stacked these images and imported them into VG STUDIOMAX v.3.3 (Volume Graphics, Heidelberg, Germany). Using the segmentation tools in VG, we isolated the skull and first presacral vertebrae as members of the subgenus *Silurana*, which includes *X. tropicalis*, are distinguished from members of the subgenus *Xenopus* by unfused nasal bones, an absent vomer, and fusion of the first two presacral vertebrae (Cannatella and Trueb, 1988; Evans et al., 2015). We uploaded all the resulting image stacks into MorphoSource (Appendix 2).

Field Sampling.—We conducted all surveys with the use of modified minnow traps, baited with chicken liver (Measey and Tinsley, 1998), excepting one pond where we relied on visual surveying because of landowner constraints. We placed traps in each respective water body around sunset and removed them the following morning, with an approximate trapping time of 12 h. The number of traps deployed at each site varied depending upon the size of the pond and the number of traps available. We deployed a minimum of 1 trap and a maximum of 30 traps per site during each sampling occasion. The large area of many ponds within the invaded area precluded us from standardizing effort across all sites. We recorded trap locations with the use of a Garmin Etrex GPS (3-m accuracy). We selected sites through the use of satellite imagery (Google Earth™). We prioritized water bodies in proximity to previous records collected by the Florida Fish and Wildlife Conservation Commission (FWC) (Hill et al., 2017) and based upon verified reports from residents in the area. Between May 2018 and October 2019, we sampled a total of 43 water bodies a minimum of two times. During each survey, we noted the life stage of any individuals of *Xenopus* observed. We designated water bodies as breeding sites if we observed any larvae of *Xenopus* during any survey. We delineated the extent of known occurrence by generating a minimum convex polygon (MCP) around all presence coordinates in ArcGIS 10.7.1 (ESRI, Redlands, CA). MCPs represent the smallest polygon around all points of interest such that no internal angle is greater than 180° (Mohr, 1947).

Results

Alignment and Phylogenetic Analyses.—Our maximum likelihood phylogeny provides strong support that the Florida population is *X. tropicalis* (Fig. 1). The average pairwise distance between our Florida population and native populations from West Africa is 0.00212, translating to a similarity of 99.79%. The average genetic diversity for all *X. tropicalis* samples ($N = 28$) is 0.4%. The low intraspecific variation among specimens from the native range precludes us from identifying a source population for the Florida population.

Micro-CT Scanning.—The five specimens scanned exhibited osteological features associated with the subgenus *Silurana*, including unfused nasal bones, a lack of vomer bones, and fusion of the first two presacral vertebrae (Fig. 2). These features preclude the possibility of this population being *X. laevis* and corroborate the analysis of DNA sequences, indicating that these represent *X. tropicalis*, which is part of *Silurana* (Evans et al., 2015).

Field Sampling.—Of the 43 water bodies sampled, we detected the presence of *X. tropicalis* in 22 of these water bodies, including the single water body where we relied on visual surveys. We observed clear signs of breeding in 11 of these water bodies based on the presence of tadpoles of *Xenopus*. Including all verified reports and data collected previously by the FWC, the total number of documented occurrences increased to 26 sites, and the number of known breeding sites increased to 12 (Fig. 3; Appendix 3). The MCP around all known occurrence points represents an area of 1,630 ha.

Discussion

Our results indicate that in contrast to previous reports (Krysko et al., 2016; Hill et al., 2017), the extant invasion of *Xenopus* in Riverview, Florida, is *X. tropicalis* and not the globally distributed nonnative species *X. laevis*. This represents the first population of *X. tropicalis* outside of its native range in West Africa. Although the vector and age of the introduction are unknown, the initial record of a *Xenopus* sp. in Hillsborough County dates back to the 1970s (Tinsley and McCoid, 1996). Researchers collected this individual near an animal import facility, close to, or within, Riverview (Godley, pers. comm.), and deposited it into scientific collections at the University of South Florida (McDiarmid, pers. comm.). Researchers initially identified this specimen as *X. laevis*. However, this was because of the species' ubiquity in global invasions rather than any formal identification (McDiarmid, pers. comm.), and an extended renovation of the University of South Florida's Science Center led to the destruction of the specimen (Mushinsky, pers. comm.). Given the proximity to currently occupied sites, this observation may represent the starting point of the extant invasion of *X. tropicalis*. This would imply an invasion detection lag of at least 34 yr. However, the imprecision of observation details and lack of any vouchered specimen make it impossible to determine the specific identity of this specimen. Indeed, both *X. laevis* and *X. tropicalis* are present in pet trade and are used in biomedical research (Beck and Slack, 2001; Measey, 2017). However, because *X. tropicalis* is diploid (opposed to tetraploid *X. laevis*; Kobel et al., 1996), it has become increasingly popular

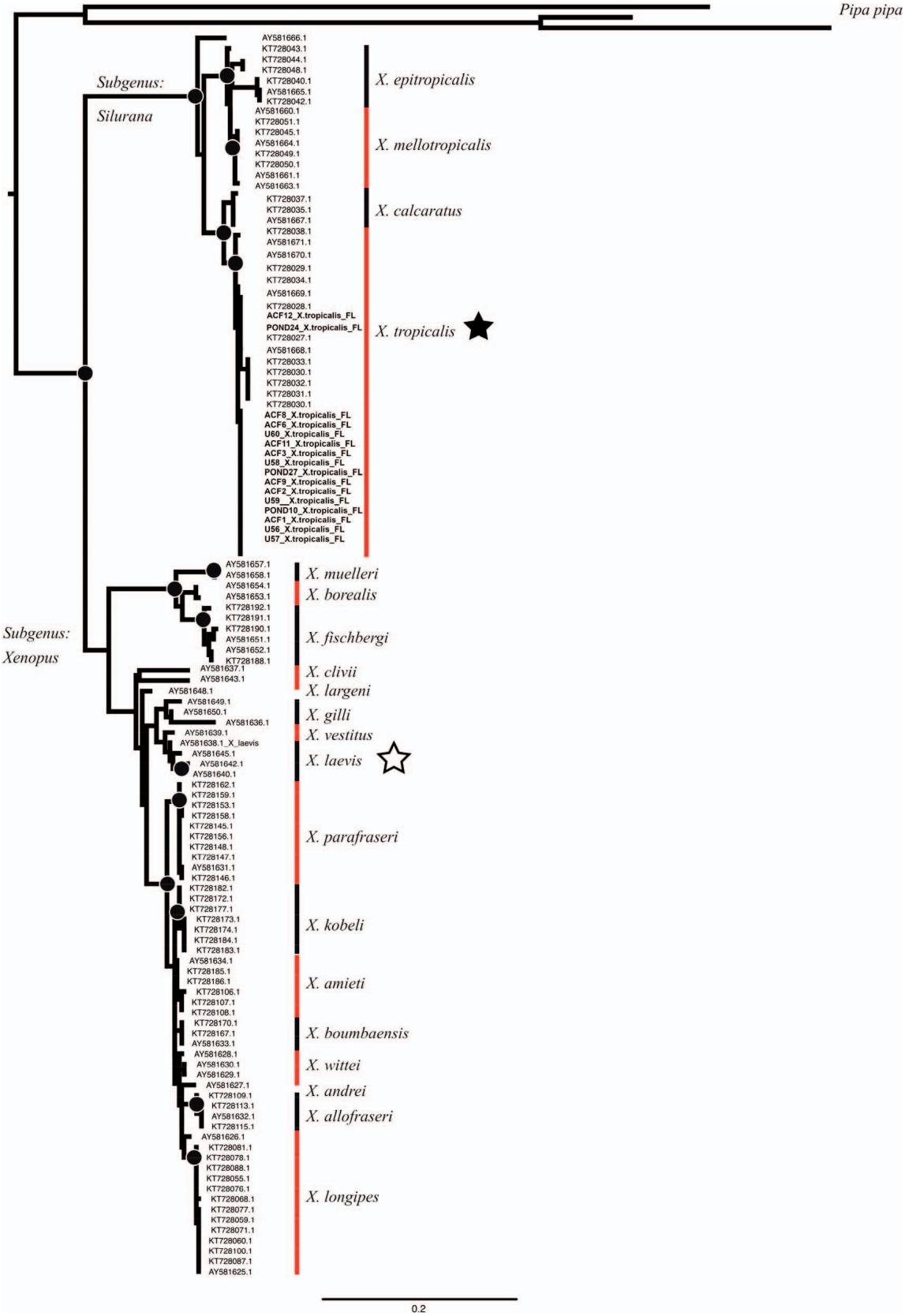

FIG. 1. Maximum likelihood phylogeny for *Xenopus* estimated using IQtree based on 16S mitochondrial sequences for all samples ($N = 119$). Black dots represent nodes with ultrafast bootstrap support >90%. Stars highlight the location of *X. tropicalis* (black star) and *X. laevis* (white star). Samples collected from within the current invaded range in central Florida are emboldened.

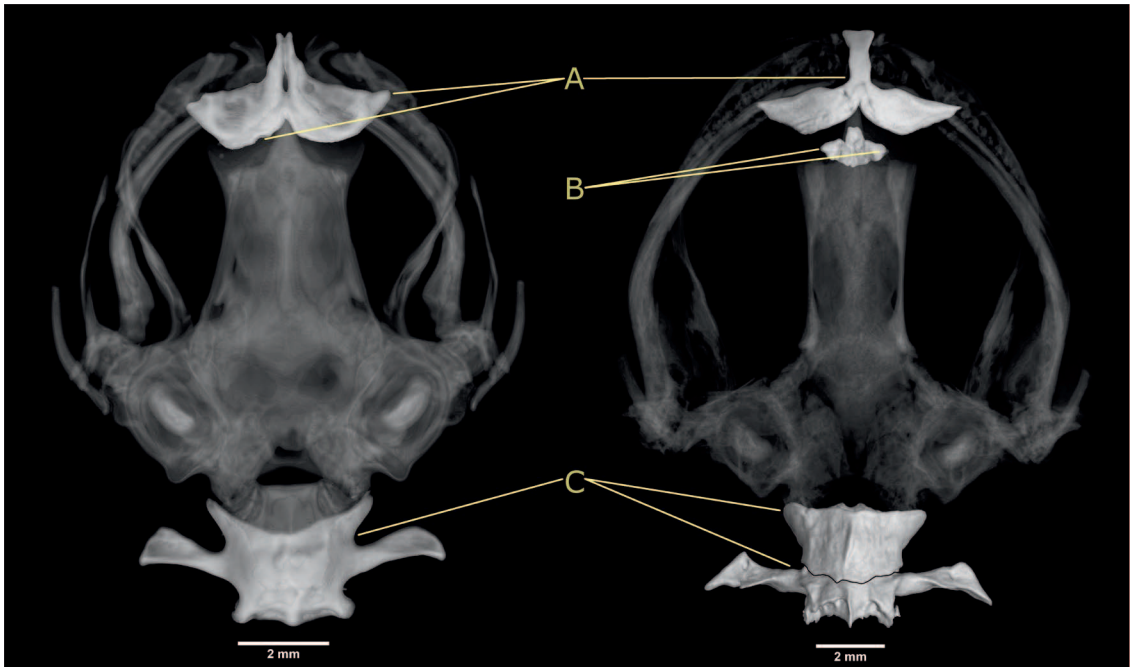

FIG. 2. Side-by-side comparison of *Xenopus tropicalis* (left, CMG-119) and *Xenopus laevis* (right, CAS:Herp:160540) based on a CT scan, illustrating three osteological traits that distinguish members of the subgenus *Silurana* from members of the subgenus *Xenopus*. (A) Unfused nasals in *Silurana*, fused nasals in *Xenopus*. (B) Absence of vomer bones in *Silurana*, presence of vomers in *Xenopus*. (C) Fusion of first two presacral vertebrae in *Silurana*, unfused presacral vertebrae in *Xenopus* (Cannatella et al., 1988). Note the difference in scales between the *X. tropicalis* and *X. laevis* specimens.

in genomics research (O'Rourke, 2007) and might possess a similar capacity for global release as *X. laevis*.

It remains unclear what impact the invasion of *X. tropicalis* in Florida is having on native species, although there is a growing body of literature on the invasive ecology of congener *X. laevis*. With at least 10 extant populations spanning four continents (Measey et al., 2012), *X. laevis* has been linked to reduced reproduction in native anurans (Lillo et al., 2011), altered macroinvertebrate composition (Courant et al., 2018a), and reduced amphibian species richness (Courant et al., 2018b). *Xenopus tropicalis* may present similar risks to Florida where native anurans are already at risk because of habitat degradation (Delis et al., 1996) and the presence of other invaders (Smith, 2005; Guzy et al., 2006). Both *X. tropicalis* and *X. laevis* are generalists, predominantly consuming aquatic macroinvertebrates (Measey, 1998; Imasuen and Aisien, 2016; Courant et al., 2017). However, both have distinct maturation rates, with *X. tropicalis* having a much shorter generation time (Hirsch et al., 2002). Generation time is an important life-history characteristic and is linked to an invasive species' ability to colonize new areas (Sakai et al., 2001). In addition, Allen et al. (2017) found that within amphibians and reptiles, species with fast life-history traits were more likely to successfully establish novel populations and to spread. It is unclear whether this disparity in life history between *X. laevis* and *X. tropicalis* will cause a concomitant disparity in the rate of invasion spread. Given the uncertainty of the source location and age of the *X. tropicalis* invasion in Florida, it is impossible to directly compare the spread rates of invasive *X. tropicalis* and *X. laevis* populations. However, even if the current *X. tropicalis* population dates to the initial vouchered specimen in 2013 (FLMNH 172054), this would still imply an annual rate of spread of <1 km, which is below the estimated rates of the French and Chilean *X. laevis* populations (Lobos and Jaksic, 2005; Fouquet and Measey, 2006).

In addition to life-history disparities, *X. tropicalis* and *X. laevis* occupy distinct thermal niches (Saito et al., 2016) and have nonoverlapping distributions in their respective native ranges (Furman et al., 2015). These differences are likely to affect the predicted habitat suitability in Florida for this *Xenopus* population. Indeed, ENMs have predicted most of Florida to be unsuitable for *X. laevis* (Measey, 2012; Rödder et al., 2017; but see Ihlow et al., 2016). Across its native and much of its invaded range, *X. laevis* is generally associated with Mediterranean or oceanic climates (Fouquet and Measey, 2006; Ihlow et al., 2016). Conversely, *X. tropicalis* is associated with tropical regions along the rain forest belt in sub-Saharan Africa (Rödel, 2000). This difference suggests that peninsular Florida may be at higher risk for further invasion of *X. tropicalis* than it would be for *X. laevis*. There have been at least three other confirmed releases of *X. laevis* in Florida (Krysko et al., 2016), including one release of ~200 individuals in the greater Miami area (King and Krakauer, 1966). Despite these releases, none have resulted in the presence of an established breeding population. Although it is impossible to rule out low number of individuals released as the cause of these failures, *X. laevis* is known to have an ability to recover from low population bottlenecks (Measey and Tinsley, 1998; Measey, 2001). This fact, coupled with low predictions of climate suitability within the area, suggests that the novel environment was at minimum a contributing factor to these failures.

Along with the potential for mismatch in predicting the rate of and ability to spread, cryptic invasions may also impact the efficacy of management efforts. One control strategy that has received localized success at extirpating *X. laevis* is the use of chemical toxicants. Wildlife managers in Lacey, Washington, appear to have successfully eradicated a small *X. laevis* population by artificially increasing the salinity of an occupied water body (Boone, 2017). Near the University of California, Davis, managers eradicated a population through the use of the insecticide Thiodan (Zacuto, 1975). At Golden Gate Park in San

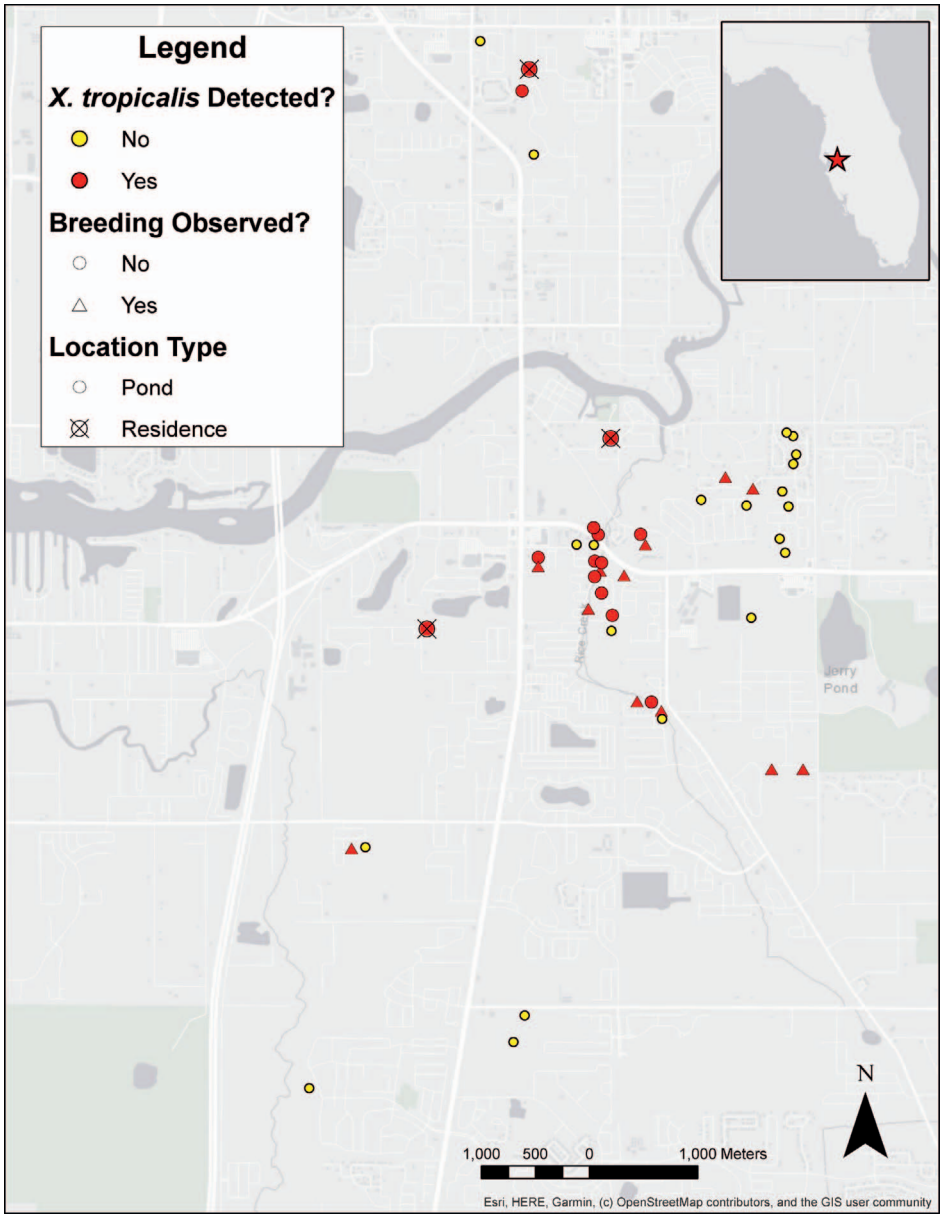

FIG. 3. Map of detections and nondetections of *Xenopus tropicalis* in Riverview, Florida, USA. Red symbols represent sites where *X. tropicalis* of any life stage were detected, yellow symbols represent sites where no *X. tropicalis* were detected, triangles represent sites where *X. tropicalis* larvae were observed, and circles represent sites where no *X. tropicalis* larvae were observed. Open objects represent ponds and cross-hatched objects represent residences. Map made in ArcGIS 10.7.1 (ESRI).

Francisco, managers were able to extirpate an isolated population by using hydrated lime, which significantly elevated the pH of both the water and sediment to a level lethal to *X. laevis* (Larson, pers. comm.). Numerous surveys have been conducted in subsequent years, and no additional *X. laevis* individuals have been detected (Larson, pers. comm.), suggesting that eradication efforts were successful. In 2016, the FWC used the same strategy in an attempt to eradicate the *X. tropicalis* invasion in Florida, treating occupied ponds with hydrated lime (Sommers, 2016). Although the treatment did appear to have an acute impact, with the population suffering high mortality, managers observed frogs at the same sites within weeks, and *X. tropicalis* still occupied all previously treated sites at the time of this study. It remains unclear whether the differences in treatment success arise from interspecific differences in toxicant susceptibility—as *X. tropicalis* and *X. laevis* last shared a common ancestor >30 million yr ago (Feng et al., 2017)—or from differences in application. Future research should seek to determine which toxicant is best suited for managing *X. tropicalis*. In addition, care should be taken before application, as any toxicant used is likely to have spillover effects on native species (Baker et al., 2013).

Although the range of *X. tropicalis* in Riverview appears to be localized, our estimated range of occurrence (1,630 ha) is based on relatively few samples and is therefore likely to be an underestimation, representing the minimum invaded range of *X. tropicalis*. Given that the genome of *X. tropicalis* is fully sequenced (Hellsten et al., 2010), future research should focus on developing methods of eDNA detection. The use of eDNA has proven reliable for the detection of congener *X. laevis* (Secondi et al., 2016) and might facilitate a more exhaustive survey in areas surrounding the known invaded range.

In conclusion, these findings underscore the importance of using contemporary methods to obtain taxonomic validation of novel invaders and present a novel opportunity to compare the invasion trajectories of two closely related species, *X. laevis* and *X. tropicalis*.

Acknowledgments.—This research was conducted with financial support from the Florida Fish and Wildlife Conservation Commission (FWC contract no. 13416-A3035). We thank D. Quinn, T. Dallas, and C. Cunningham of FWC for providing preliminary data and C. Watson and staff at the University of Florida, Institute of Food and Agricultural Sciences Tropical Aquaculture Laboratory for providing material support. Thanks to N. Claunch and O. Nathan for offering editorial suggestions. We also thank two reviewers for their helpful comments on this article. This study's methods comply with national and international guidelines and received Institutional Animal Care and Use Committee (IACUC) approval (University of Florida IACUC no. 201810274). This study adheres to the Association for the Study of Animal Behaviour/Animal Behavior Society Guidelines for the Use of Animals in Research. DNA sequence data are available at GenBank (accession nos. MT345772–MT345787). CT scan data are available through www.morphosource.org (see Appendix 2).

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Accepted: 2 December 2020.
Published online: 19 March 2021.

Appendix 1. ID, GenBank accession number, and location of collection for all *Xenopus tropicalis* samples used in PCR. All locations are in WGS84 Datum.

Field ID	GenBank accession no.	Collection latitude	Collection longitude
ACF12_*X.tropicalis*_FL	MT345786	27.83278	−82.34078
POND24_*X.tropicalis*_FL	MT345787	27.85622	−82.32018
ACF8_*X.tropicalis*_FL	MT345785	27.85054	−82.31909
ACF6_*X.tropicalis*_FL	MT345775	27.85309	−82.31815
U60_*X.tropicalis*_FL	MT345784	27.85309	−82.31815
ACF11_*X.tropicalis*_FL	MT345777	27.85553	−82.31630
ACF3_*X.tropicalis*_FL	MT345774	27.84384	−82.31552
U58_*X.tropicalis*_FL	MT345782	27.84384	−82.31552
POND27_*X.tropicalis*_FL	MT345779	27.85694	−82.32008
ACF9_*X.tropicalis*_FL	MT345776	27.85965	−82.30758
ACF2_*X.tropicalis*_FL	MT345773	27.85047	−82.32113
U59_*X.tropicalis*_FL	MT345783	27.85309	−82.31815
POND10_*X.tropicalis*_FL	MT345778	27.85198	−82.31998
ACF1_*X.tropicalis*_FL	MT345772	27.85366	−82.32003
U56_*X.tropicalis*_FL	MT345780	27.85366	−82.32003
U57_*X.tropicalis*_FL	MT345781	27.85366	−82.32003

APPENDIX 2. Information on the *Xenopus tropicalis* specimens used for CT scans, including sex, mass (g), measurements of snout–vent length (SVL), and each specimen's corresponding MorphoSource specimen ID. All locations are in WGS84 Datum.

Field ID	MorphoSource specimen ID	Collection latitude	Collection longitude	Sex	Mass (g)	SVL (mm)
CMG-110	30541	27.85363	−82.32002	Female	8.21	43.78
CMG-111	30544	27.85363	−82.32002	Male	5.38	39.08
CMG-119	30540	27.85363	−82.32002	Female	12.94	49.65
CMG-125	30543	27.85353	−82.31797	Male	9.52	44.16
CMG-128	30539	27.85309	−82.31838	Male	6.64	39.70

APPENDIX 3. Locations of all known sites where individuals of *Xenopus tropicalis* were detected. Locations named "Residence" represent confirmed reports from citizens and not ponds. All locations are in WGS84 Datum.

Site ID	Latitude	Longitude	*X. tropicalis* detected	Breeding detected	Location type
Site_01	27.85367	−82.32007	Yes	Yes	Pond
Site_02	27.85085	−82.32104	Yes	Yes	Pond
Site_03	27.84406	−82.31699	Yes	Yes	Pond
Site_04	27.84338	−82.31498	Yes	Yes	Pond
Site_05	27.85331	−82.31808	Yes	Yes	Pond
Site_06	27.85969	−82.30738	Yes	Yes	Pond
Site_07	27.86054	−82.30967	Yes	Yes	Pond
Site_08	27.85558	−82.31631	Yes	Yes	Pond
Site_09	27.83326	−82.34072	Yes	Yes	Pond
Site_10	27.85399	−82.32521	Yes	Yes	Pond
Site_11	27.83909	−82.30322	Yes	Yes	Pond
Site_12	27.84402	−82.3158	Yes	No	Pond
Site_13	27.85038	−82.31905	Yes	No	Pond
Site_14	27.85202	−82.31995	Yes	No	Pond
Site_15	27.85322	−82.32053	Yes	No	Pond
Site_16	27.85437	−82.32051	Yes	No	Pond
Site_17	27.85631	−82.32023	Yes	No	Pond
Site_18	27.85463	−82.3252	Yes	No	Pond
Site_19	27.85682	−82.32061	Yes	No	Pond
Site_20	27.85633	−82.3167	Yes	No	Pond
Site_21	27.85424	−82.31995	Yes	No	Pond
Site_22	27.88894	−82.32655	Yes	No	Pond
Site_23	27.84925	−82.31913	No	No	Pond
Site_24	27.86355	−82.30404	No	No	Pond
Site_25	27.86381	−82.30458	No	No	Pond
Site_26	27.8615	−82.30403	No	No	Pond
Site_27	27.85498	−82.3047	No	No	Pond
Site_28	27.85601	−82.30515	No	No	Pond
Site_29	27.85949	−82.30495	No	No	Pond
Site_30	27.85558	−82.32202	No	No	Pond
Site_31	27.85555	−82.32059	No	No	Pond
Site_32	27.85838	−82.30443	No	No	Pond
Site_33	27.8622	−82.30378	No	No	Pond
Site_34	27.83335	−82.33956	No	No	Pond
Site_35	27.81564	−82.34422	No	No	Pond
Site_36	27.81904	−82.32726	No	No	Pond
Site_37	27.82098	−82.32633	No	No	Pond
Site_38	27.84278	−82.31493	No	No	Pond
Site_39	27.85887	−82.31167	No	No	Pond
Site_40	27.85021	−82.30752	No	No	Pond
Site_41	27.85845	−82.30792	No	No	Pond
Site_42	27.8926	−82.33002	No	No	Pond
Site_43	27.88427	−82.32557	No	No	Pond
Site_44	27.83909	−82.30583	Yes	Yes	Pond
Site_45	27.89054	−82.32594	Yes	No	Residence
Site_46	27.8494	−82.33446	Yes	No	Residence
Site_47	27.86339	−82.31917	Yes	No	Residence