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EDITED BY
Amanda Kissel,
United States Geological Survey, United States

REVIEWED BY
Steve A. Johnson,
University of Florida, United States
Andrew Parks,
US Fish and Wildlife Service, United States

*CORRESPONDENCE
Erin B. Brosnan
erin.brosnan@ucf.edu

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Tracing invasion routes of Cuban treefrogs into Louisiana using mitochondrial DNA

Erin B. Brosnan[1]*, Karen A. Paniagua Torres[2], Katherine R. Martin[1], Matthew S. Atkinson[1], Brad M. Glorioso[3], J. Hardin Waddle[4], Robert W. Mendyk[5] and Anna E. Savage[1]

[1]Department of Biology, University of Central Florida, Orlando, FL, United States, [2]Department of Biology, The Pennsylvania State University, University Park, PA, United States, [3]U.S. Geological Survey, Wetland and Aquatic Research Center, Lafayette, LA, United States, [4]U.S. Geological Survey, Wetland and Aquatic Research Center, Gainesville, FL, United States, [5]Department of Herpetology, Audubon Zoo, New Orleans, LA, United States

Understanding the origin and spread of invasive species is critical for predicting when and where new introductions will establish, and impact native species. However, due to the complexity of contributing factors such as multiple introductions, dispersal method, genetic admixture in founding populations, and variable propagule pressure, genetic patterns observed in invasive species may not always conform to a single theoretical expectation. Cuban treefrogs (*Osteopilus septentrionalis*) are invasive in peninsular Florida and sporadically in the Florida panhandle. Though *O. septentrionalis* has been occasionally reported in Louisiana since the 1990s, established populations were not present until the discovery of a breeding population in New Orleans in 2017. In this study we investigated the source of this novel population using existing and newly generated cytochrome B (cyt-b) mitochondrial gene sequences from the native and invasive range of *O. septentrionalis*. We recovered a total of 14 cyt-b haplotypes, nine novel and five previously published. Within the 95 Louisiana invasion samples, we recovered seven haplotypes including five novel haplotypes. The haplotypes most common in Louisiana were shared exclusively with west and east Florida localities in central Florida, indicating a possible source population. The presence of haplotypes private to the Louisiana locality suggests other unsampled localities may also be contributing to the Louisiana settlement. Metrics of genetic diversity across native and invasive localities did not significantly differ. Furthermore, the Louisiana samples had higher genetic diversity than any single location sampled within Florida. Thus, genetic diversity and our haplotype connectivity suggest the Louisiana population is derived from multiple introductions from Florida. Our study highlights how demographic and genetic analyses can be utilized to understand the source and future expansion potential of invasive populations.

KEYWORDS

invasive species, genetics, biological invasion, cytochrome b, *Osteopilus septentrionalis*

1 Introduction

Invasive herpetofauna pose a threat to biodiversity through disruption of native species interactions (Rice et al., 2011; Richter-Boix et al., 2013; Culbertson and Herrmann, 2019; Hill et al., 2019), displacement of native species through predation (Dove et al., 2011; Piquet and López-Darias, 2021), and the spread of infectious diseases (Rivera et al., 2019; Atkinson and Savage, 2023). While over 780 introductions of non-native herpetofauna were documented globally as of 2015, only 250 species have established as independent populations (Kraus, 2015). The disparity between introduced and established invasive species underscores the importance of life history, environmental variation, propagule pressure and genetic diversity on the ability of an invasive population to establish and thrive. Typically, generalist species are more likely to successfully invade and establish compared to specialists that have more specific habitat requirements (Pitt et al., 2005), and environments more like the invasive species native range tend to be conducive to establishment (Bomford et al., 2009). Introductions of non-native herpetofauna largely result from anthropomorphic activity, such as the pet trade (Willson et al., 2011), farming (Laufer et al., 2008), bio-control efforts (Shanmuganathan et al., 2010), and stowaways in cargo shipments (Richmond et al., 2015). Thus, the numerous factors influencing which species become introduced combined with the complexity of novel ecosystems present a challenge for predicting whether and which non-native amphibian and reptile species will become established.

Invasive populations can arise from novel founder events or expansions of pre-existing invasive ranges, and each of these mechanisms should produce distinct patterns of genetic diversity in the invasive population. Founder events are generally expected to decrease genetic diversity and increase inbreeding depression (Frankham, 1998), which has been observed in the case of the coqui (*Eleutherodactylus coqui*) invasion in Hawaii (Peacock et al., 2009) and the American bullfrog (*Rana catesbeiana*) invasion in China (Bai et al., 2012). Contrary to this expectation, invasive populations founded by multiple introduction events or with high propagule pressure (i.e., many individuals introduced in one or more release events) (Rius and Darling, 2014) can maintain genetic diversity at adaptive loci (Selechnik et al., 2019; LaFond et al., 2022) or even show higher overall genetic diversity relative to native populations (Allendorf and Lundquist, 2003; Kolbe et al., 2004). A genetically admixed invasive population has increased adaptive potential and often is capable of faster range expansions (Wagner et al., 2017) and adaptation to novel environmental conditions (Krehenwinkel and Tautz, 2013; Rius and Darling, 2014).

Mitochondrial DNA (mtDNA) is commonly used to establish phylogenetic relationships between populations and trace the origins of invasive populations (Avise et al., 1987; Hamner et al., 2007; Colangelo et al., 2015) because it has a higher mutation rate than nuclear loci, allowing for increased resolution of genetic variation (Gray et al., 1989). Additionally, mtDNA is easy to isolate, has a relatively simple genetic structure (i.e., lacking transposable elements, pseudogenes and introns) and exhibits a straightforward mode of inheritance (Avise et al., 1987). The mitochondrial gene cytochrome b (cyt-b) codes for a central redox catalytic subunit involved in the electron transport chain that is present in nearly all eukaryotic organisms. The cyt-b gene is used to evaluate genetic diversity, trace geographic origins, and distinguish cryptic invasive species across vertebrate groups (Kochzius et al., 2003; Hamner et al., 2007; Colangelo et al., 2015; Jackson et al., 2015). Cyt-b data have been used to investigate the origins of several invasive herpetofauna, including the novel invasion of two subspecies of *Boa constrictor* in Puerto Rico (Reynolds et al., 2013), the origin and identity of cryptic *Xenopus* species in Chile (Lobos et al., 2014) and Italy (Lillo et al., 2013), the invasion of the greenhouse frog (*Eleutherodactylus planirostris*) in Florida (Heinicke et al., 2011) and China (Hong et al., 2022), and the invasive American bullfrog (*R. catesbeiana*) across the western United States (Bai et al., 2012; Kamath et al., 2016; LaFond et al., 2022).

The Cuban treefrog (*Osteopilus septentrionalis*) is one of the most successful invasive amphibians in the state of Florida (Meshaka, 2001). Invasive populations negatively influence native Florida treefrogs through predation (Wyatt and Forys, 2004), and by influencing pathogen dynamics (Galt et al., 2021; Atkinson and Savage, 2023). The native *O. septentrionalis* range spans Cuba, the Cayman Islands and the Bahamas, with invasive populations first establishing in the Florida Keys in the late 1800s to early 1900s (Duellman and Schwartz, 1958; Heinicke et al., 2011). Subsequently, *O. septentrionalis* spread to peninsular Florida by the mid-1900s via multiple anthropogenic introductions (Heinicke et al., 2011) and has now spread throughout much of central and northern Florida and in some areas of the Florida panhandle (Figure 1; Johnson, 2023). Sporadic sightings of *O. septentrionalis* have been recorded along the southern coastline of Alabama and Mississippi (Morningstar et al., 2024), but no established populations have been reported. Cyt-b from *O. septentrionalis* has been used to evaluate the ecological drivers of haplotype and species diversity (Rodríguez et al., 2015). Akin to the previous herpetological invasions mentioned above, the origin of invasive *O. septentrionalis* in Florida was investigated using cyt-b genetic analysis (Heinicke et al., 2011). The resulting mitochondrial phylogeny revealed a deep phylogenetic split among haplotypes of *O. septentrionalis*, with both clades represented throughout the native range in Cuba and the invasive range in Florida. This finding indicated that current populations are descended from at least two source populations (Heinicke et al., 2011).

In 2017, the first established breeding population of *O. septentrionalis* in Louisiana, USA, was observed in New Orleans (Glorioso et al., 2016, 2018c). Although *O. septentrionalis* had been sporadically documented in Louisiana since the 1990s, no breeding colonies had been observed prior to this event (Chatfield and Vance, 2014; Glorioso et al., 2018b, 2018c). In contrast, the discovery of individuals in different age classes, ranging from tadpoles to metamorphs, were found in Audubon Zoo and the adjacent Riverview Park beginning in 2016, leading to further investigation of this invasion event (Glorioso et al., 2018c). Subsequently, a second breeding population was located in St. Charles Parish

(Glorioso et al., 2018a). While the novel New Orleans invasion coincided with the arrival of palms that were brought from Lake Placid, Florida for an Audubon Zoo exhibit (Glorioso et al., 2018c), given the occasional observations of *O. septentrionalis* prior to this event, and that *O. septentrionalis* are regularly dispersed via anthropogenic activities (Palis et al., 2021), a definitive source for this invasion remains unclear.

Here, we used cyt-b mitochondrial genetics to evaluate genetic variation in the recently established *O. septentrionalis* population at the Riverview Park and St. Charles Parish sites in Louisiana and assess likely routes of introduction. We sampled and generated cyt-b haplotypes from 31 east Florida individuals and 40 west Florida individuals within central Florida, and 95 individuals in New Orleans, Louisiana, then combined these new data with previously generated sequences from throughout the native and invasive range (Heinicke et al., 2011; Rodríguez et al., 2015). We conducted phylogenetic and haplotype network analyses and compared metrics of genetic diversity in Louisiana to other native and invasive populations. We used these data to assess whether the Louisiana invasion represents a single introduction event or a more complex history of population establishment.

2 Materials and methods

We collected and processed a total of 166 *O. septentrionalis* between 2017 and 2022. Of those, 31 were from east Florida, 40 from west Florida, and 95 individuals were from Louisiana populations. East Florida samples were collected from one site in Orange County and west Florida samples were collected from one site in Hillsborough County. Louisianan samples originated from two sampling sites; 85 individuals were from Audubon Riverview Park and the adjacent Audubon Zoo in Orleans Parish, while 10 individuals were from 15 kilometers west in St. Charles Parish (Figure 1).

2.1 Louisiana field sampling

As a means to collect *O. septentrionalis*, artificial PVC refugia were set at the Riverview Park site in 2019 to capture juvenile metamorphs and adults (Glorioso and Waddle, 2014). Additional samples were collected by hand via standardized visual surveys. Toe clips from metamorphosed anurans were collected in 99%

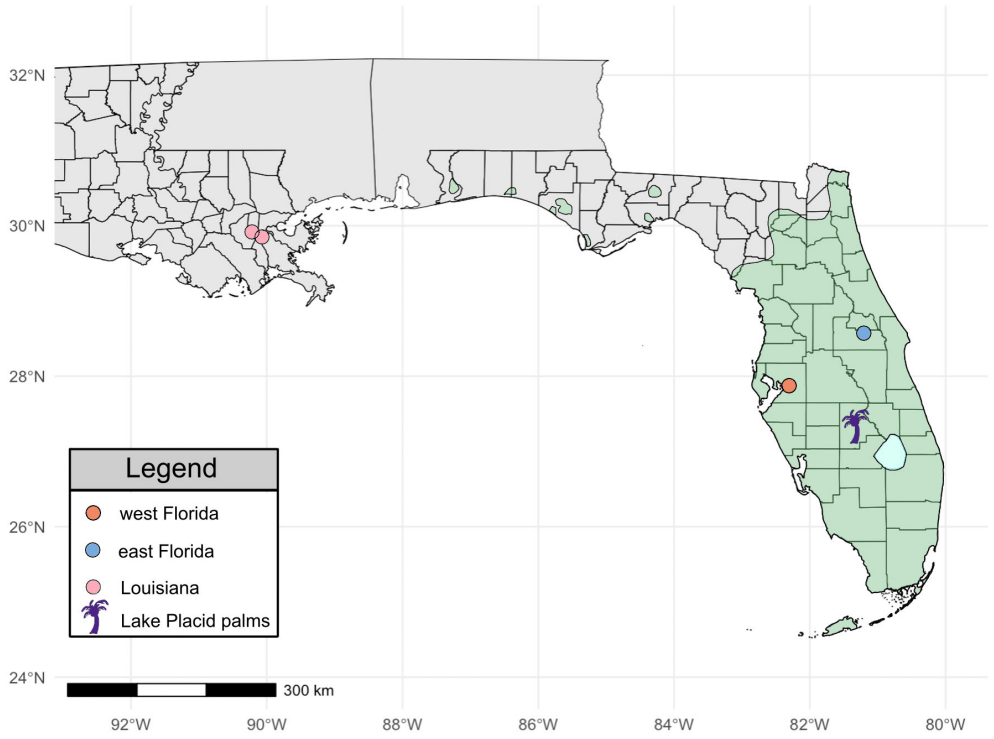

FIGURE 1
Louisiana and Florida *O. septentrionalis* sampling locations. Sampling locations are denoted with circles. Louisiana populations are in pink, east Florida samples in blue, and west Florida samples in orange. Lake Placid, the origin of Audubon Zoo palms, is denoted with a purple palm. Green shading signifies the known *O. septentrionalis* distribution as of January 2023 (Credits: Tracey Bryant, UF/IFAS). Light blue shading represents Lake Okeechobee.

molecular grade ethanol. Scissors were sterilized after each toe clip by alcohol immersion. *O. septentrionalis* were euthanized via 20% benzocaine. Samples were placed in 99% molecular grade ethanol and stored at -80 °C prior to DNA extraction.

2.2 Florida field sampling

Juvenile metamorphs and adult frog samples were collected by hand via standardized visual surveys. Tadpoles were collected via standardized dip netting and minnow trapping. Tail clips from tadpoles and toe clips from metamorphosed anurans were collected with flame sterilized scissors and placed in 99% molecular grade ethanol. Per Florida regulations on invasive species, *O. septentrionalis* were euthanized via MS-222 (tricane mesylate). Tissues were preserved in 99% molecular grade ethanol and stored at -20 °C prior to DNA extraction.

2.3 Laboratory methods

We performed DNA extractions on toe or tail tissue samples using a Qiagen DNeasy Blood and Tissue Kit (Qiagen Corporation, Germantown, Maryland, USA) following manufacturer instructions. Extractions were stored at -20 °C prior to further molecular analysis. PCR was performed on all samples to amplify the cyt-b mitochondrial gene using the forward and reverse primers CBL17 (5'-TAGCCTTYTCATCCGTYGCCCATAT-3') and CBH18 (5'-GTTGATAATGCAACCCTRACCCGATT-3') (Heinicke et al., 2011). Each 25μL reaction consisted of 16.4μL of molecular grade water, 5μL of NEB OneTaq Buffer, 0.5μL of 10mM dNTPs, 0.5μL each of 10uM forward and reverse primers, 0.13μL of NEB One Taq DNA polymerase and 2μL of template DNA or controls. Thermocycling conditions for PCR consisted of an initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of 94 °C for 30 seconds, 46 °C for 30 seconds, and 72 °C for 60 seconds, with a final elongation step of 72 °C for one minute (Heinicke et al., 2011). PCR products were sent to Eurofins Genomics LLC for Sanger sequencing in the forward and reverse direction. Our newly generated sequences were trimmed of primers and manually inspected and edited to resolve errors and ambiguities by analyzing sanger sequencing graphic outputs. Any sequences that were shorter than 670 bp or had ambiguities, such as stop codons, were excluded from the analyses.

2.4 Phylogenetic analyses

We reconstructed a Bayesian gene tree of *O. septentrionalis* cyt-b using our sequences (N = 166) and a mix of sequences from native and invasive populations available in GenBank (N = 210; Heinicke et al., 2011; Rodríguez et al., 2015). We used *Rana septentrionalis* (AY083273), *Hyla molleri* (MK172098), *Osteopilus marianae* (HQ831741), *Osteopilus vastus* (HQ831742), *Osteopilus dominicensis* (HQ831743), and *Osteopilus ocellatus* (HQ831744) as outgroups. The untruncated sequences of 903 bp were aligned using Clustal Omega (Sievers et al., 2011) with default parameters in Geneious v. 9.0.5 software (Kearse et al., 2012). To find a model of evolution that best fit the data and to use in the phylogenetic reconstruction, we used jmodeltest2 v. 2.1.1 (Darriba et al., 2012) on the CIPRES Science Gateway server (Miller et al., 2010). The best model according to the Akaike information criterion for small sample sizes (AICc) was the Hasegawa-Kishino-Yano (HKY) model with invariant sites and a gamma distribution. We reconstructed a Bayesian gene tree using MrBayes v. 3.2.7a (Ronquist et al., 2012) on the CIPRES Science Gateway server (Miller et al., 2010). The phylogenetic reconstruction consisted of two independent runs of 1.0×10^7 generations and four chains each. We sampled every 500th generation and the first 100,000 generations were discarded as burnin. We used Tracer v. 1.7 (Rambaut et al., 2018) to confirm Markov chain Monte Carlo convergence and adequate sampling of the posterior distribution. We also reconstructed a maximum likelihood gene tree with IQ-TREE with 1000 ultrafast bootstraps and SH-aLRT branch tests on the IQ-TREE webserver (Trifinopoulos et al., 2016) to compare its topology to that of MrBayes. We visualized the Bayesian gene tree and geographic locations where each haplotype has been found using the R package ggtree v3.3.0.900 in RStudio using R v. 4.1.2 (Yu et al., 2017; RStudio Team, 2021).

2.5 Haplotype network

We reconstructed a haplotype network of *O. septentrionalis* cyt-b utilizing all sequences we generated from our west and east Florida sampling, as well as all additional sequences sampled from Florida *O. septentrionalis* that were available in GenBank, resulting in the addition of a south Florida locality. Sequences in our alignment were truncated to produce uniform 712 bp long sequences. Cyt-b sequences generated in previous studies that were shorter than 330 bp were excluded from the haplotype network to preserve the quality of our analysis. We used the program PopArt v. 1.7 (Leigh and Bryant, 2015) and the TCS algorithm (Clement et al., 2000) to visualize haplotype connectivity and distribution with a haplotype network analysis.

2.6 Genetic diversity

Untruncated sequence lengths of 903 bp were used to compare genetic diversity among native and invasive locations utilizing our east and west Florida samples, and all *O. septentrionalis* that were available in GenBank. We separated cyt-b samples into those occurring in Louisiana, central Florida, west Florida, south Florida, west Cuba, central Cuba, east Cuba, and the Caribbean. For each location, we calculated the number of segregating sites (S) and nucleotide diversity (pi) for the preceding locations using pegas v. 1.2 (Paradis, 2010) and ape v. 5.7.1 (Paradis and Schliep, 2019) in RStudio. We generated the distribution of pi with 95% confidence intervals by employing a bootstrap resampling technique with

TABLE 1 Cytochrome B (cyt-b) mitochondrial haplotypes recovered from our sampling efforts.

Haplotype	Frequency (n)	Localities		
		Louisiana	*west Florida*	*east Florida*
se3	19	18	1	
se7	1		1	
se13	7		2	5
se17	1			1
se29	8	1	2	5
se86*	81	36	29	16
se87*	38	37		1
se88*	4		4	
se89*	1	1		
se90*	1	1		
se91*	2			2
se92*	1			1
se93*	1		1	
se94*	1	1		

Haplotypes with a star (*) are novel to this study.

10,000 iterations using the boot function from boot package v. 1.3.28.1 in R (Davison and Hinkley, 1997; Canty, 2021). For reproducibility, we set the random number generation seed to 500 using the set.seed function in R.

3 Results

We generated robust cyt-b sequences from all 166 frogs we sampled in west Florida, east Florida, and Louisiana, recovering 14 total haplotypes. Of these, nine are novel (GenBank Accession numbers: PP320231.1 – PP320239.1) and five were previously published (Heinicke et al., 2011; Table 1). Novel haplotype se86 was the most common sequence found in our sampling, occurring in 49% of all frogs (Table 1).

In Louisiana we recovered seven haplotypes from 95 individuals, including two previously published haplotypes (Table 1) and five novel haplotypes. Among the five novel haplotypes, se89, se90, and se94 were private alleles recovered exclusively from Louisiana, each from a single individual (Table 1). Novel haplotype se87 was the most common haplotype in Louisiana (N = 37 individuals) and it was also found in one east Florida frog. Novel haplotype se86 was the second most common haplotype in Louisiana (N = 36 individuals), and we also found it at high frequency in west and east Florida (Figure 2). All ten individuals collected from the St. Charles Parish population had this haplotype. Haplotypes se3 (N = 17) and se29 (N = 1) were recovered exclusively in Florida in previous studies. None of the haplotypes present in Louisiana occurred in frogs from Cuba or the Caribbean (electronic supplemental material, Supplementary Table S1).

The 31 frogs we sampled from east Florida had seven haplotypes, including four novel haplotypes (se92, se92, se86, se87) and three previously published haplotypes (se13, se17, se29). Only two individuals had the novel private haplotype se91, and one individual had the novel private haplotype se92. Most east Florida individuals had the novel haplotype se86 (N = 16), which was also found in west Florida and Louisiana. One east Florida individual had the novel haplotype se87, which was the most common haplotype in Louisiana. The three haplotypes se13, se17, and se29 were previously recovered from west Florida and west Cuba, south Florida and the Caribbean, and west Florida and south Florida, respectively (electronic supplemental material, Supplementary Table S1). Here, we recovered all three of these haplotypes the first time in east Florida.

In west Florida, we recovered seven haplotypes from 40 individuals: three novel haplotypes (se86, se88, se93) and four previously published haplotypes (se3, se7, se13, se29). A single individual had the private novel haplotype se93 and four individuals had the private novel haplotype se88. The novel haplotype se86 was the most common haplotype in west Florida, similar to its high frequency in Louisiana and east Florida. Se3 was previously recovered only in west Florida, but here we found it in one west Florida frog and in 17 Louisiana frogs. We found se7 in west Florida for the first time, but only in one frog. This haplotype was previously found in a single south Florida individual (electronic supplemental material, Supplementary Table S1). We found two other low-frequency haplotypes (se13 and se29) that had previously been detected in Florida and Cuba (electronic supplemental material, Supplementary Table S1).

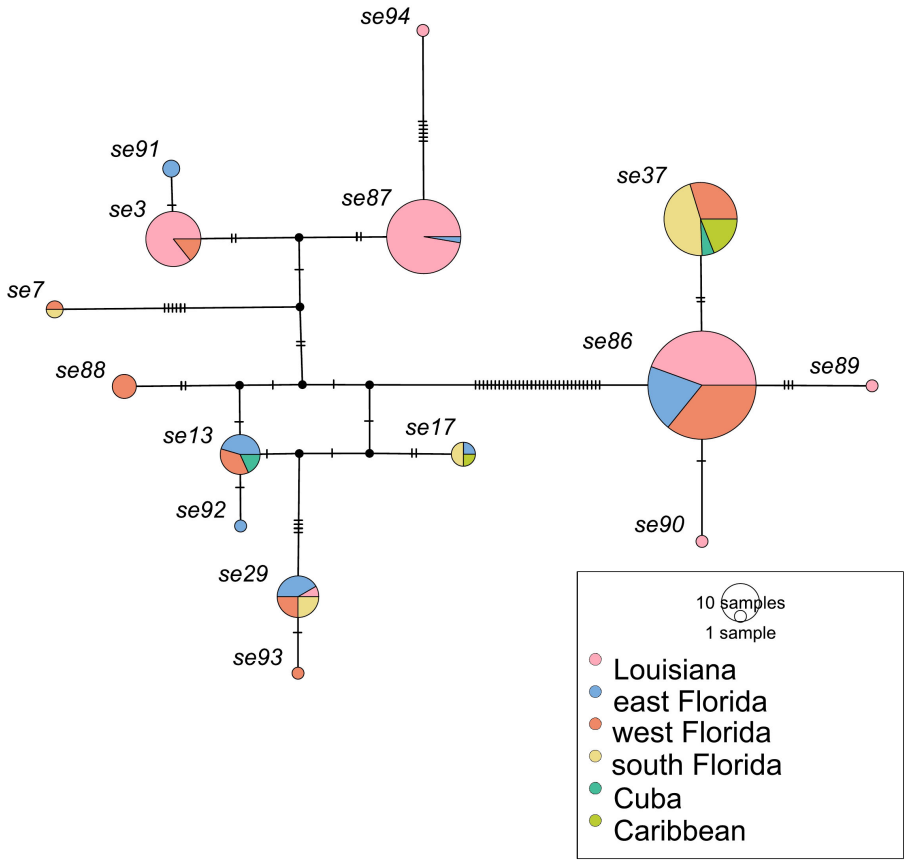

FIGURE 2
Cyt-b haplotype network of all sequences recovered in *O. septentrionalis* individuals sampled in Florida and Louisiana, USA. Circle size corresponds to sample size and color denotes sampling location. Tick marks between haplotypes represent the number of nucleotide changes between haplotypes.

Our haplotype network illustrates that almost all frogs sampled in Louisiana had haplotypes se3, se86, or se87 (Figure 2). These sequences are only found in Louisiana, east Florida (se86 and se87), and west Florida (se3 and se86). Only one Louisiana frog had a haplotype that is recovered more broadly throughout Florida (se29), and this sequence was many mutational steps away from all other haplotypes found in Louisiana. Private Louisiana haplotypes se89 and se90 were respectively three and one mutational steps away from se86. Likewise, private Louisiana haplotype se94 is six mutational steps from se87, a haplotype only rarely found outside of Louisiana. Collectively these patterns suggest the evolution of new haplotypes within the invasive Florida and/or Louisiana populations.

Phylogenetic analysis of *O. septentrionalis* mitochondrial sequences revealed minimal geographic structure (Figure 3). We recovered a well-supported basal split between two sequences only found in the Caribbean and all other sequences. The next split was also well-supported and separated one sequence private to Cuba with everything else. All other sequences were separated into two large clades with moderate support (posterior probability = 0.8917). Both of these clades contained numerous haplotypes found in multiple localities through the native and invasive range. Haplotypes sampled from Louisiana frogs were dispersed throughout the phylogeny.

Among all known cyt-b sequences, Cuba contained the highest number of haplotypes. Frogs sampled in west Cuba had more haplotypes than any other location, followed by east Cuba and central Cuba (Table 2). The Caribbean, Louisiana, and Florida localities each contained similar numbers of haplotypes (Table 2). Native Cuban localities contained similar percentages of private haplotypes, which were high in comparison to the invasive range. Private haplotypes made up 20–30% of the haplotypes recovered in the invasive range localities of east Florida, west Florida, and south Florida whereas 42.86% of Louisiana haplotypes were private (Table 2). Nucleotide diversity in Louisiana was highest among invasive locations in the United States (Figure 4). Nucleotide diversity in Louisiana (0.039; 95% CI = 0.033–0.056) did not overlap with nucleotide diversity of central Cuba (0.017; 95% CI = 0.0092–0.021) or east Cuba (0.018; 95% CI = 0.010–0.023; Table 2; Figure 4). Segregating sites were higher in west Cuba and the Caribbean than within invasive localities in the United States (Table 2).

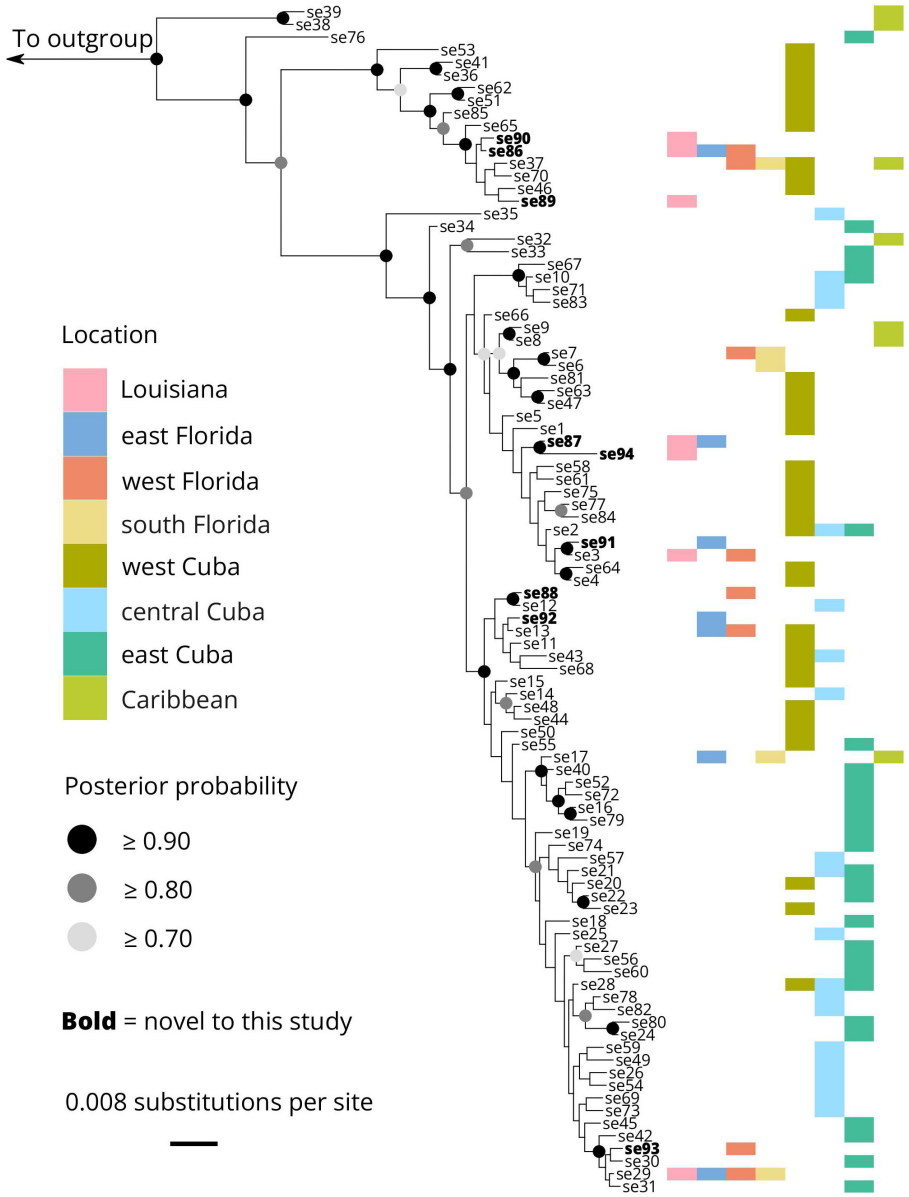

FIGURE 3
Bayesian gene genealogy of cytochrome-b in *O. septentrionalis*. Colored horizontal bars denote the different geographic locations where samples were taken. Haplotypes se1 through se39 originated from Heinicke et al. (2011). Haplotypes se40 through se85 originated from Rodríguez et al. (2015). Haplotypes se86 through se94 originate from this study and are denoted in bold.

4 Discussion

Our study is the first to investigate the origins of the establishment of *O. septentrionalis* in Louisiana, the first documented establishment of *O. septentrionalis* in the contiguous U.S. outside of Florida (Glorioso et al., 2018c). We aimed to determine whether this invasion was the result of one or more founding events from remote locations. Our results indicate both Louisiana *O. septentrionalis* populations are the result of multiple introduction events, likely from central Florida localities. Four of seven haplotypes found in Louisiana individuals were present only in Florida and Louisiana, while the remaining three were rare and only found in Louisiana. This pattern excludes Caribbean locations as direct sources of the novel population and strongly points toward Florida origins. One haplotype lending the most support to our introduction hypothesis is se86, which is novel to our study (Table 1). It was the most abundant haplotype we recovered overall and was at high frequency in our Florida and Louisiana sample sites, consistent with the hypothesis that central Florida is a source of the Louisiana *O. septentrionalis*.

Though we did not sample any localities in the Florida panhandle, the likelihood that frogs spread naturally from Florida to Louisiana in a range expansion is minimal. The high nucleotide diversity of haplotypes in Louisiana is inconsistent with the expectation for a range expansion. Range expansions of invasive species typically result in decreased genetic diversity as the leading

TABLE 2 Summary of allelic diversity, private alleles, segregating sites, and nucleotide diversity across eight locations.

Location (No. frogs sampled)	No. haplotypes	No. private haplotypes	Percentage of private haplotypes	No. segregating sites	Nucleotide diversity (standard error)
Louisiana (95)	7	3	42.86%	60	0.039 (± 0.02214)
east Florida (31)	7	2	28.57%	54	0.024 (± 0.01378)
west Florida (56)	8	2	25.00%	67	0.032 (± 0.01761)
south Florida (24)	5	1	20.00%	62	0.032 (± 0.01975)
west Cuba (70)	36	29	80.56%	86	0.040 (± 0.01949)
central Cuba (29)	20	15	75.00%	54	0.017 (± 0.00900)
east Cuba (55)	28	20	71.43%	67	0.018 (± 0.00927)
Caribbean (16)	7	5	71.43%	99	0.048 (± 0.08660)

edge expands (Wilson et al., 2005; Davis et al., 2011). Instead, we see higher nucleotide diversity in Louisiana compared to all locations except for west Cuba and the Caribbean (Figure 4), which is where the species originated (Heinicke et al., 2011; Johnson, 2023). Notably, the nucleotide diversity in Louisiana is nearly equivalent to values found in west Cuba. Furthermore, there is no evidence for a leading edge, as established populations of *O. septentrionalis* between the Florida panhandle and the Louisiana population have not yet been documented (Johnson, 2023). Future work that samples *O. septentrionalis* from the Florida panhandle and the Gulf Coast of Alabama and Mississippi, and analyzes their genetic composition could enable assessment of potential new population establishments and identification of their source populations.

High genetic diversity metrics in the Louisiana population suggests a high propagule pressure invasion and/or multiple introduction events (Kolbe et al., 2008; Simberloff, 2009; Wang et al., 2019). Given that human transport is the most plausible route of invasion, multiple founding events is the most likely scenario explaining the patterns we recovered in Louisiana. For example, repeated transport of one or a few individuals traveling on ornamental plants purchased from locations within Florida and planted in New Orleans is a plausible source of the novel invasion. Anecdotal accounts of *O. septentrionalis* being found within many purchased plants from Florida (Johnson, 2023), the scale and economic importance of the nursery industry in Florida (Khachatryan et al., 2022), and low efficacy of state-level biosecurity measures in preventing the spread of established invasive species (Paini et al., 2010) lend support to this hypothesis. Therefore, it is plausible that the known planting of ornamental palms from Lake Placid, Florida in the Audubon Zoo contributed to this invasion (Glorioso et al., 2018c). Sampling in or around the nursery in Lake Placid—which lies farther south than our sampling sites—to verify the presence of haplotypes shared with Louisiana could strengthen support for this hypothesis. Additionally, increased sampling at or near ornamental horticultural nurseries and retailers in top economic contributing

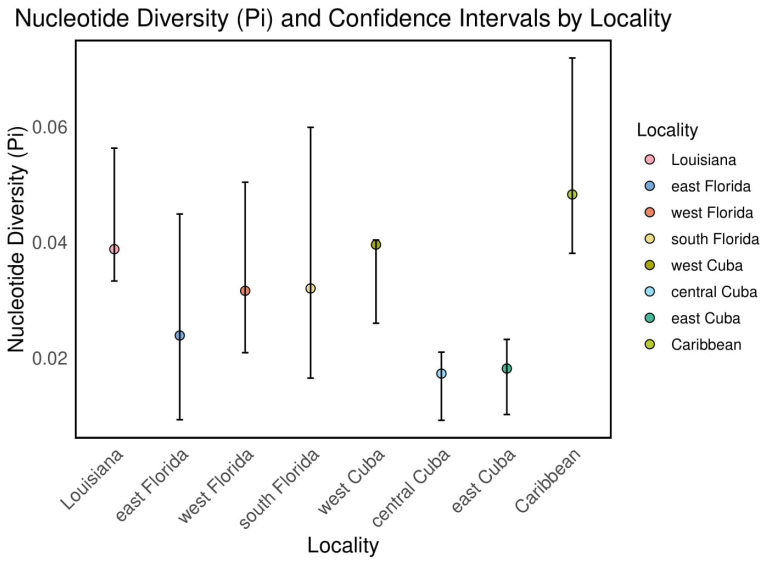

FIGURE 4
Nucleotide diversity (pi) of *O. septentrionalis* by locality with 95% confidence intervals.

regions, such as central and south Florida (Hodges et al., 2016), could give greater insight into the impacts of this industry on the spread of *O. septentrionalis*.

Human traffic near the invasion site has the potential to facilitate introductions from distant source populations. New Orleans is a large city that is visited by nearly 20 million tourists annually, with many visitors arriving via vehicular transportation or large cruise ships that depart from docks approximately 5 miles from the invasion site (Adams, 2024). Florida is a frequent point of origin, ranking as the 3rd most common origin of interstate travel entering Louisiana in 2023 (Adams, 2024). As vehicular transport is the primary mode of invasion of *O. septentrionalis* (Johnson, 2023), it is plausible that multiple instances of individual frogs hitch-hiking within vehicles departing from central Florida have contributed to the New Orleans invasion. Additionally, Interstate 10 (I-10), a major thoroughfare linking Florida to Louisiana, provides direct access to New Orleans and is heavily utilized by Floridians during hurricane evacuations (Ghorbanzadeh et al., 2021). While hurricane evacuees likely are minor contributors in comparison to tourists, increasing hurricane severity and resulting evacuation efforts may increase the frequency of these individual translocation events. Additionally, cruise ships departing and arriving at the Port of New Orleans frequent many Caribbean islands and various Floridian ports. Caribbean sampling of *O. septentrionalis* is minimal, leaving unclear resolution on what haplotypes are present in this region. Increased Caribbean sampling may reveal haplotypes from this study found exclusively in Louisiana may also occur in the Caribbean and may be derived from there. Overall increased sampling efforts throughout Florida, as well as the Caribbean could provide increased clarity on the contribution of translocation events to the founding of the Louisianan population.

Heinicke et al. (2011) previously reported two divergent clades of *O. septentrionalis* to be phylogeographically grouped based on relation to the Guanahacabibes peninsula in remote west Cuba. They additionally reported Bahamian haplotypes form a basal monophyletic group. With the additional samples of Rodríguez et al. (2015) as well as our novel sequences, our phylogenetic analysis groups together west Cuban haplotypes within and outside of the Guanahacabibes peninsula together, indicating this peninsular boundary does not impede gene flow to other localities in Cuba. Our study does not expand on the Heinicke et al., 2011 finding of Bahamian samples forming a monophyly, as we did not include additional Bahamian sampling outside of Heinicke et al. (2011) providing another reason for additional sampling in the Caribbean and specifically the Bahamas.

We expect that *O. septentrionalis* will continue to negatively affect native species through resource use and influences on local pathogen dynamics, but changes in the genetic composition of *O. septentrionalis* populations are a potential cause for concern. Anecdotal sightings of *O. septentrionalis* outside their established invasive territory are plentiful and range from Georgia to as far north as Vermont along the east coast of the United States (Morningstar et al., 2024). These sightings are likely a result of anthropogenic transport either via horticulture or other items transported from their invasive range to wider regions of the U.S. and are not yet representative of established breeding colonies. Cold temperatures play a large role in limiting the northern distribution of *O. septentrionalis*, which have been observed dead at temperatures below 0 °C (Haggerty and Crisman, 2015), but shifting global temperatures threaten this climatic barrier to expansion. With fewer freeze events occurring in regions north of the current range of *O. septentrionalis*, these populations may persist and establish farther north than previously expected (Osland et al., 2021).

Admixed populations at range limits may also benefit from an increased evolutionary potential resulting from heterosis, thereby increasing the extent of dispersal and probability of permanent establishment (Forsman, 2013; Wagner et al., 2017). Novel genotypes resulting from heterosis (Verhoeven et al., 2011) may allow for adaptation to colder temperatures. Evidence shows *O. septentrionalis* from the northern latitudinal limits of their Florida range had critical thermal minima trending lower than their southern counterparts and that in general *O. septentrionalis* has a higher tolerance for cold weather than previously believed (Simpson, 2013). Though cold tolerance tends to result from phenotypic plasticity (McCann et al., 2014; Mittan and Zamudio, 2019), it is possible that exposure to freeze events and the existence of novel genotypes within northern populations result in adapted cold tolerance. Though we observed patterns suggesting local adaptation of the cyt-b mitochondrial gene in Florida and Louisiana, more work could help to assess adaptation in functionally relevant genes. Future experimental studies on mitochondrial gene transcription associated with thermal regulation across invasive populations could provide deeper insight into thermal adaptation (Hong et al., 2024).

Disparities in size between northern and southern populations of *O. septentrionalis* have also been recorded (McGarrity and Johnson, 2009). Studies do not yet indicate if this shift to smaller sizes in colder latitudes is genetically based or if these changes result from environmental effects such as shifts in food availability or metabolic constraints resulting from cold temperatures. It is possible that smaller body size and increasing urban development may aid in their spread, as *O. septentrionalis* can exploit urban refugia to escape or minimize exposure to thermal temperature limits (Haggerty and Crisman, 2015; Meshaka, 2001).

Invasive species present a unique opportunity to study evolution in action by assessing novel selection pressures *in situ*. These systems can serve as natural experiments, with introduced populations functioning as treatment groups and native populations acting as controls. Invasive *O. septentrionalis* have the potential to serve as a powerful model for studying rapid adaptation to novel environmental conditions, especially in the recently established Louisiana population. While mitochondrial genes markers have historically served as a valuable tool in determining the origin of invasive species, next-generation sequencing (NGS) techniques offer better resolution to fine scale population structure and more robust findings on this recent population divergence (North et al., 2021). Whole mitochondrial genome analysis can expand on findings derived from single genes

for more comprehensive insights as demonstrated with *Rhinella marina*, the invasive cane toad (Cheung et al., 2024). Like many amphibian species, *O. septrentrionalis* has not yet had its nuclear or mitochondrial genome sequenced, due in part to the difficulty of assemblage (Kosch et al., 2024; O'Connell et al., 2024) inhibiting the depth of evolutionary questioning utilizing this species.

Our study concludes that the newly established Louisiana population of *O. septentrionalis* is derived from multiple introductions, primarily from central Florida. These introductions likely occurred via anthropogenic means (Johnson, 2023), though we cannot conclude with certainty whether the transport of flora from Florida to Louisiana contributed to this establishment. To fully resolve the origin of Louisiana's population, future work could prioritize increased sampling across the Florida panhandle, Caribbean, and key Floridian localities. By identifying the source populations and the degree of admixture within the Louisiana population, we gain insight into where management efforts could be prioritized and understanding of the genetic variation available for selection to act upon. In the case of *O. septentrionalis*, this knowledge can be used not only for reconstructing invasion history, but could also anticipate the species' capacity to adapt to colder climates, urban environments, and other novel pressures.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

EB: Investigation, Writing – review & editing, Visualization, Formal analysis, Project administration, Writing – original draft, Data curation. KP: Data curation, Investigation, Writing – review & editing. KM: Investigation, Writing – review & editing, Formal analysis, Visualization, Data curation, Project administration. MA: Data curation, Project administration, Investigation, Funding acquisition, Writing – review & editing, Writing – original draft, Conceptualization. BG: Methodology, Investigation, Resources, Data curation, Project administration, Funding acquisition, Conceptualization, Writing – review & editing. JW: Conceptualization, Methodology, Resources, Data curation, Investigation, Writing – review & editing, Project administration, Funding acquisition. RM: Investigation, Data curation, Writing – review & editing, Resources. AS: Writing – review & editing, Funding acquisition, Investigation, Supervision, Resources, Project administration, Methodology.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/famrs.2025.1646714/full#supplementary-material

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