

# GENETIC CHARACTERIZATION OF THE CUBAN TREEFROG (*Osteopilus septentrionalis*) IN PUERTO RICO USING MITOCHONDRIAL COI GENE DNA BARCODES THROUGH NON-INVASIVE METHODS

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## BACKGROUND

- Biological invasions are among the leading causes of biodiversity loss worldwide and can alter ecosystem structure, species interactions, and ecological processes (Wake & Vredenburg, 2008; Everts et al., 2025).
- The Cuban Treefrog (*Osteopilus septentrionalis*) is a large arboreal amphibian native to Cuba, the Cayman Islands, and the Bahamas that has successfully established introduced populations throughout the Caribbean and other regions (Meshaka, 2001; Ríos-López, 2021).
- In Puerto Rico, *O. septentrionalis* was first reported in 1978 and has since expanded throughout much of the island, becoming one of the most successful introduced amphibian species in urban, agricultural, and natural habitats (Joglar, 1998; Ríos-López, 2021).
- The species exhibits high ecological plasticity and is capable of occupying a wide variety of natural and human-modified environments, contributing to its invasion success (Joglar, 1998; Ríos-López, 2021).
- Introduced amphibians may affect native biodiversity through predation, competition, disease transmission, and alterations of ecological interactions (Wake & Vredenburg, 2008; Everts et al., 2025).
- DNA barcoding using the mitochondrial cytochrome c oxidase subunit I (COI) gene provides a reliable tool for species identification and the study of genetic variation in invasive species (Hebert et al., 2003; Che et al., 2012; Ratnasingham & Hebert, 2013).



## RATIONALE

- Nearly five decades have passed since the introduction of *O. septentrionalis* to Puerto Rico, providing sufficient time for founder effects, genetic drift, local adaptation, and other evolutionary processes to influence the genetic composition of island populations (Zhang et al., 2024; Everts et al., 2025).
- Once established outside their native range, invasive species may experience genetic changes that differ from those present in their original populations, potentially influencing dispersal, adaptation, and invasion success (Zhang et al., 2024).
- Genetic analyses have proven valuable for reconstructing invasion histories, identifying introduction pathways, evaluating population connectivity, and understanding dispersal dynamics in invasive amphibians (Zhang et al., 2024; Everts et al., 2025).
- Understanding the genetic variation of Puerto Rican populations of *O. septentrionalis* may provide insights into the species' invasion history and contribute to future assessments of its ecological impact on native amphibian communities and local biodiversity (Joglar, 1998; Ríos-López, 2021).
- Generating COI barcode data for Puerto Rican populations of *O. septentrionalis* will strengthen regional genetic databases and provide a foundation for future studies on invasion biology, population genetics, and biodiversity conservation.

## OBJECTIVES

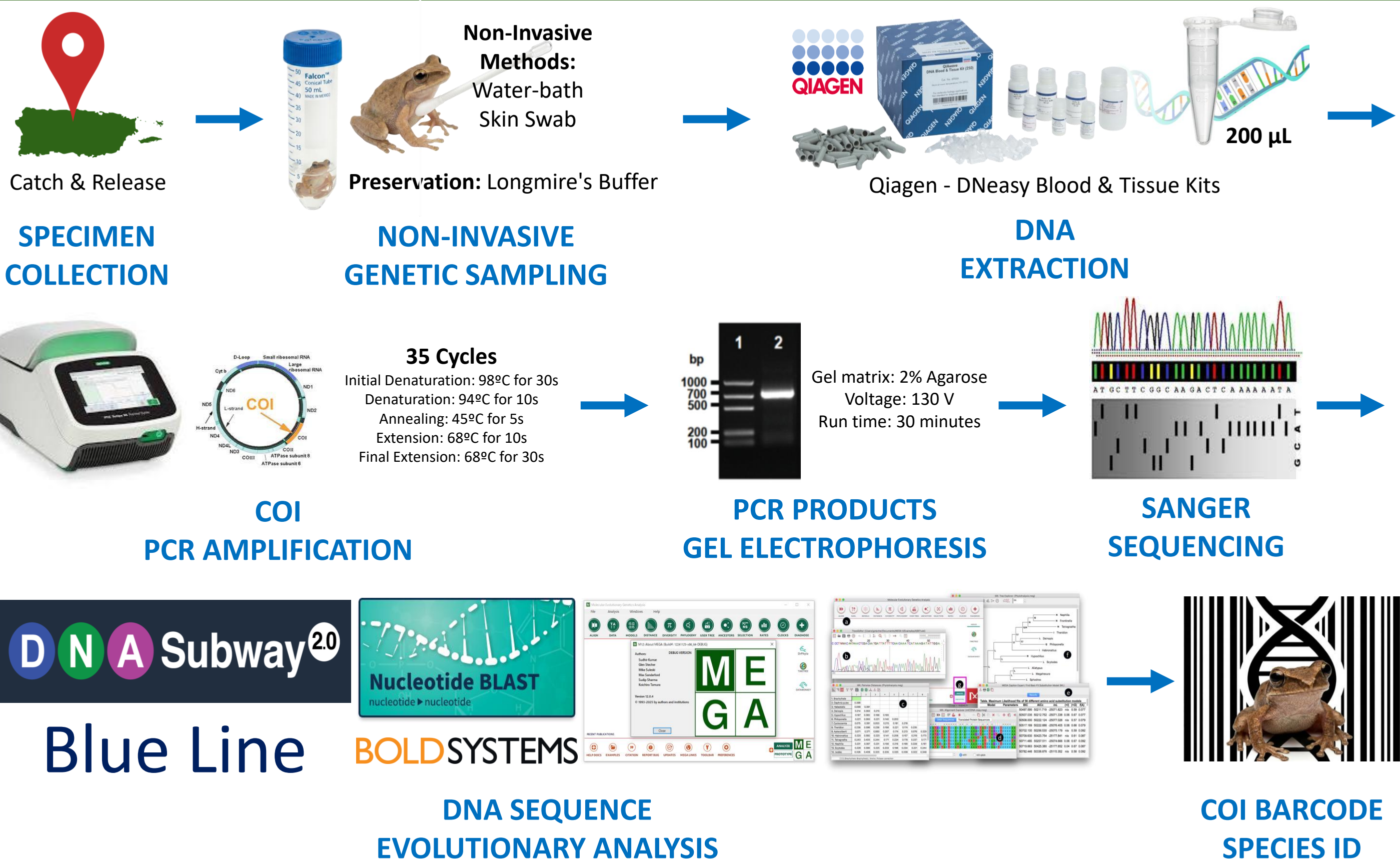
### General Objective

- Characterize the genetic variation of Puerto Rican populations of *O. septentrionalis* using mitochondrial COI DNA barcodes obtained through non-invasive sampling methods.

### Specific Objectives

- Collect genetic material from *O. septentrionalis* using non-invasive sampling techniques.
- Generate and analyze mitochondrial COI sequences for species confirmation through DNA barcoding.
- Compare COI sequences among individuals from different localities in Puerto Rico and identify patterns of genetic variation.
- Evaluate evidence of genetic differentiation among Puerto Rican populations of *O. septentrionalis*.
- Contribute COI barcode data that may support future studies on invasion dynamics, population connectivity, and conservation of Puerto Rican biodiversity.

## MATERIALS AND METHODS



## COLLECTED INDIVIDUALS

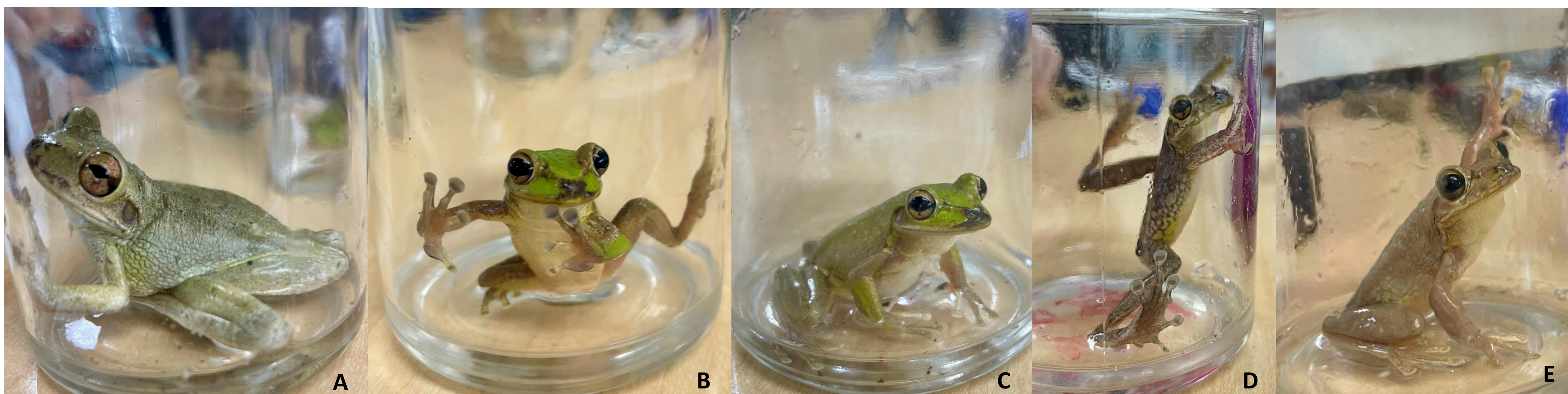


Figure. Representative *O. septentrionalis* (Cuban Treefrog) individuals collected for non-invasive genetic sampling in Puerto Rico. A) Adult female collected in Cidra, Puerto Rico. B-E) Adult males collected in Cayey, Puerto Rico. Individuals were sampled using non-invasive methods, including water-bath and dermal swab protocols, prior to molecular analyses. The figure illustrates variation in coloration and external morphology among sampled individuals. All animal handling and sampling procedures were conducted under UPR-Río Piedras IACUC Protocol No. 2026-01-22 MB5.

## SAMPLING METHODS



Figure. Non-invasive sampling procedures used for genetic analyses of *O. septentrionalis*. A) Capture of an individual using a hand-held fish net. B-C) Individuals undergoing the water-bath sampling procedure, in which frogs were temporarily placed in sterile water to collect shed DNA. D-F) Dermal swab sampling procedure, showing the collection of epithelial cells from the skin surface using a sterile swab. Both methods were used to obtain DNA samples for subsequent COI amplification and DNA barcoding analyses.

## RESULTS

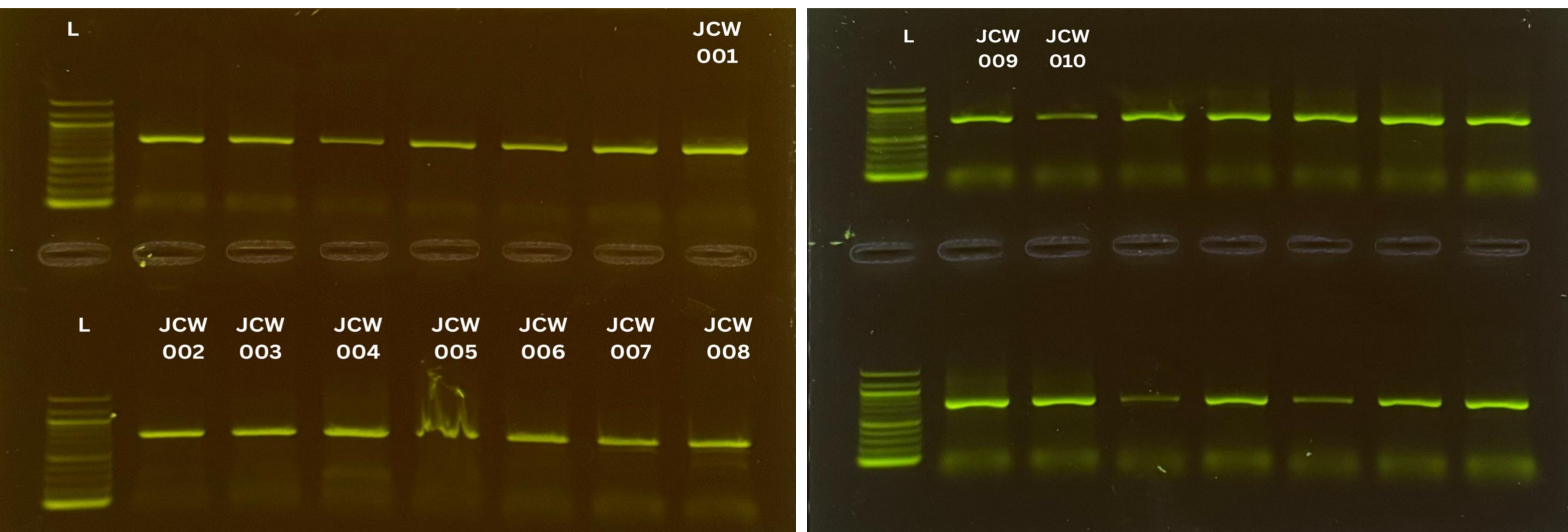


Figure. Agarose gel electrophoresis of COI gene PCR amplification products. Lane L represents the 100 bp DNA ladder. Lanes labeled JCW 001 through JCW 010, demonstrate amplification expected amplicon. All labeled sample lanes exhibit a distinct, single band corresponding to the expected PCR product size of approximately 650 bp. Sample JCW 005 shows a slight migration distortion (smiling effect), without compromising the specificity of the amplicon.

Table. Mean DNA concentration (ng/μL) obtained from *O. septentrionalis* individuals collected in Puerto Rico using two non-invasive sampling methods: water bath (WB) and dermal swab (S). DNA concentrations were measured using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific), and values represent the average of three independent readings obtained from 1 μL of DNA extract per measurement. Individual sample codes and sex are provided for each specimen. The reported concentrations should be interpreted considering the instrument's reported accuracy and the inherent variability associated with low-concentration DNA samples.

| x̄ DNA Concentration (ng/μL) |     |             |     |             |      |
|------------------------------|-----|-------------|-----|-------------|------|
| Species                      | Sex | Sample Code | WB  | Sample Code | S    |
| <i>O. septentrionalis</i>    | F   | JWC-001     | 5.5 | JWC-006     | 7.2  |
|                              |     | JWC-002     | 6.1 | JWC-007     | 8.1  |
|                              |     | JWC-003     | 5.6 | JWC-008     | 13.1 |
|                              | M   | JWC-004     | 7.2 | JWC-009     | 7.8  |
|                              |     | JWC-005     | 4.0 | JWC-010     | 11.8 |
|                              |     | x̄          | 5.7 | x̄          | 9.6  |

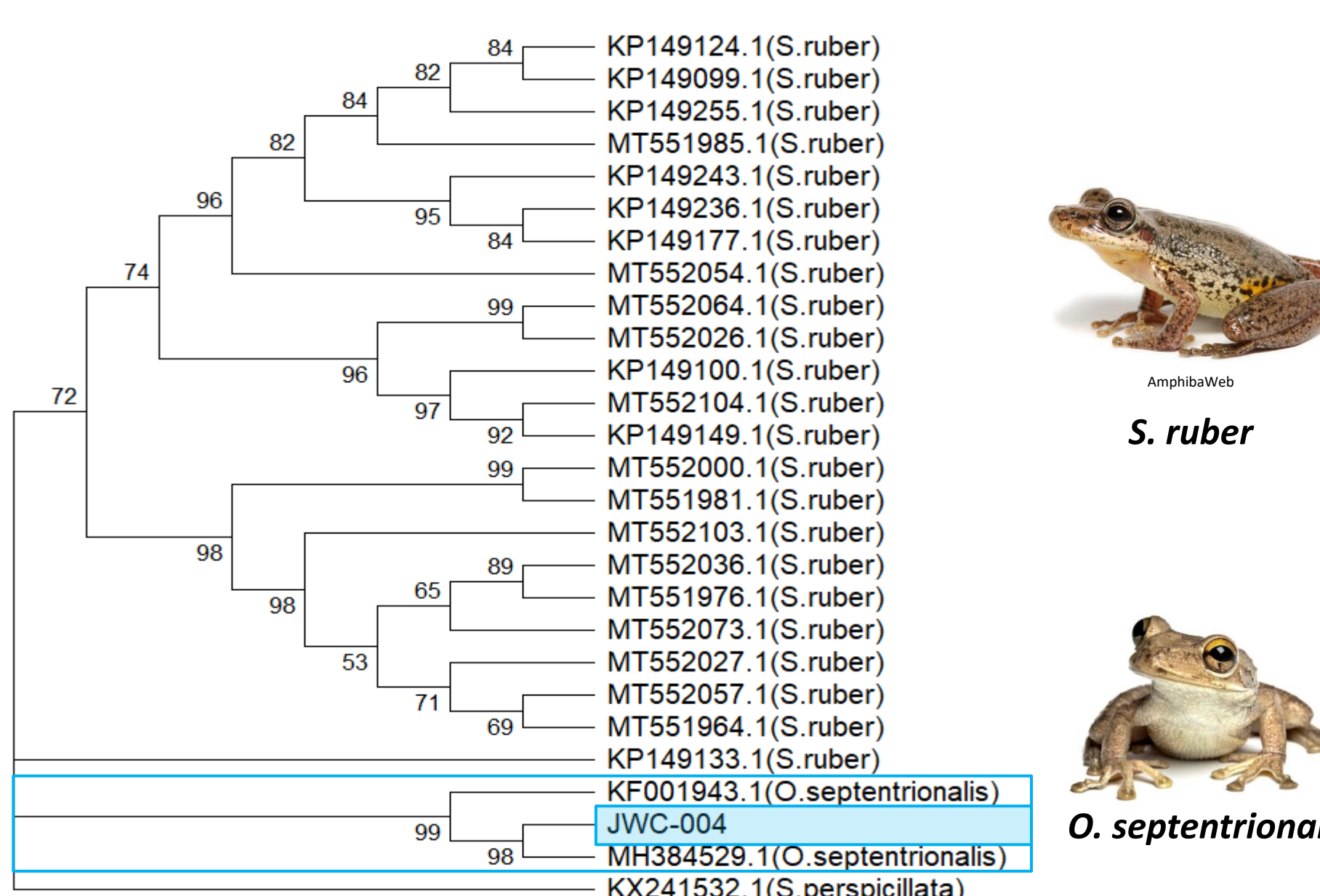


Figure. Neighbor-Joining phylogenetic tree based on mitochondrial COI sequences of *O. septentrionalis* and related hylid frogs. Sample JCW-004 clustered with reference sequences of *O. septentrionalis* with strong bootstrap support (99%), confirming its species identity. Sequences of *Scinax ruber* were included for comparison, and *Salamandrina perspicillata* was used as the outgroup. Bootstrap values (>50%) are shown at the nodes.

## DISCUSSION

- Although all samples produced PCR products of the expected size (~650 bp), only two sequences matched *O. septentrionalis* in BLAST analyses, and only one sequence (JWC-004) was retained for phylogenetic reconstruction. This highlights the difficulty of recovering high-quality target COI sequences from non-invasive samples (Che et al., 2012; Brustenga et al., 2024).
- Several sequences matched non-target organisms, including *Pseudomonas spp.* and other taxa, suggesting co-amplification of environmental and microbial DNA. Similar challenges have been reported when highly degenerate COI primers are used in complex biological samples (Che et al., 2012; Leese et al., 2021).
- The CHMF4/CHMR4 primers were designed to maximize amplification across amphibian taxa (Che et al., 2012). While advantageous for DNA barcoding, their degeneracy may increase amplification of non-target DNA when target DNA is scarce (Leese et al., 2021).
- Although dermal swabs produced higher average DNA concentrations (9.6 ng/μL) than water-bath samples (5.7 ng/μL), only water-bath samples generated sequences matching *O. septentrionalis*, suggesting that DNA concentration alone may not predict sequencing success (Brustenga et al., 2024).
- Sample JWC-004 clustered with *O. septentrionalis* reference sequences with strong bootstrap support (99%), confirming species identity and demonstrating the utility of COI DNA barcoding for invasive amphibian identification (Hebert et al., 2003; Che et al., 2012).
- Sample JWC-005 also matched *O. septentrionalis* but exhibited substantially greater sequence divergence (119–139 mismatches) than JWC-004 (23–45 mismatches). Due to its lower sequence quality, JWC-005 was excluded from phylogenetic analyses (Kumar et al., 2024).
- Interpretation of genetic variation remains limited because only three *O. septentrionalis* COI reference sequences are currently available in GenBank, restricting evaluation of population structure and introduction history (Ratnasingham & Hebert, 2013).
- Nearly five decades after its introduction into Puerto Rico, *O. septentrionalis* may have experienced founder effects, genetic drift, and local adaptation. Similar genetic changes have been documented in other invasive amphibians and may contribute to divergence from source populations over time (Zhang et al., 2024; Everts et al., 2025).
- These preliminary results provide baseline genetic information for one of Puerto Rico's most successful invasive amphibians and support future studies of invasion dynamics, population connectivity, and biodiversity conservation (Ratnasingham & Hebert, 2013; Zhang et al., 2024; Everts et al., 2025).

## FUTURE WORK

- Expand sampling to additional populations across Puerto Rico to better assess genetic variation.
- Generate and deposit additional COI sequences in public databases to improve reference coverage for *O. septentrionalis*.
- Evaluate alternative PCR conditions, polymerases, and primer sets to improve sequencing success and reduce non-target amplification.
- Compare Puerto Rican populations with individuals from the native range to investigate potential genetic changes associated with invasion and establishment.
- Develop a genetic reference library to support future studies on invasion dynamics, population connectivity, and biodiversity conservation.

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