MOLECULAR IDENTIFICATION OF AMPHIBIAN TADPOLES FROM PUERTO RICO USING COI GENE DNA BARCODES THROUGH NON-INVASIVE SAMPLING

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BACKGROUND

- Amphibians are vital ecosystem indicators facing severe global declines, making accurate identification across all life stages essential for monitoring and conservation (Wake & Vredenburg, 2008).
- Unlike adults, tadpoles exhibit highly conserved morphology, rendering visual identification based on external characters extremely difficult and prone to error.
- At least eight amphibian species share local aquatic breeding environments, including the endemic *Peltophryne lemur*, the native *Leptodactylus albilabris*, and introduced/invasive species such as *Rhinella marina, Lithobates grylio*, *Aquariana catesbeianus*, *Scinax ruber,* and *Osteopilus septentrionalis*.
- The physical overlap among these tadpoles hinders both the monitoring of protected species and the early detection of invasive threats (Rivero, 1978; Joglar, 1998).
- DNA barcoding with the mitochondrial COI gene bypasses visual limitations, definitively linking identical-looking tadpoles to characterized adult species (Vences et al., 2005; Che et al., 2012).

RATIONALE

- Accurate larval identification is essential for understanding population dynamics, reproductive success, and species distribution, particularly in insular regions like Puerto Rico with high diversity and endemism.
- Identifying tadpoles using only physical traits is severely limited due to high external similarity among species, developmental changes, and shared reproductive habitats, which can lead to critical errors in ecological studies.
- The coexistence of native, endemic, and introduced species producing visually identical tadpoles creates a major knowledge gap. The inability to distinguish protected species (like *P. lemur*) from invasive threats (like *R.marina*, *A. catesbeianus*, or *O.septentrionalis*) hinders population monitoring and aquatic management (Rivero, 1978; Joglar, 1998).
- DNA barcoding using the mitochondrial COI gene is an internationally established standard that effectively resolves these taxonomic ambiguities (Vences et al., 2005; Che et al., 2012), directly addressing the current lack of genetic reference data for Puerto Rican tadpoles.
- Generating genetic data for larval stages enables the identification of active breeding areas and the early detection of invasive species before changes become evident in adult populations, which is vital for native biodiversity (Wake & Vredenburg, 2008).
- The use of non-invasive DNA collection methods adheres to ethical research principles, allowing for sufficient genetic recovery while minimizing stress, injury, and long-term impacts on vulnerable organisms.
- This project is justified by the urgent need to establish a scientifically validated, ethically responsible methodological framework that strengthens genetic databases and improves taxonomic accuracy for the island's herpetofauna.

OBJECTIVES

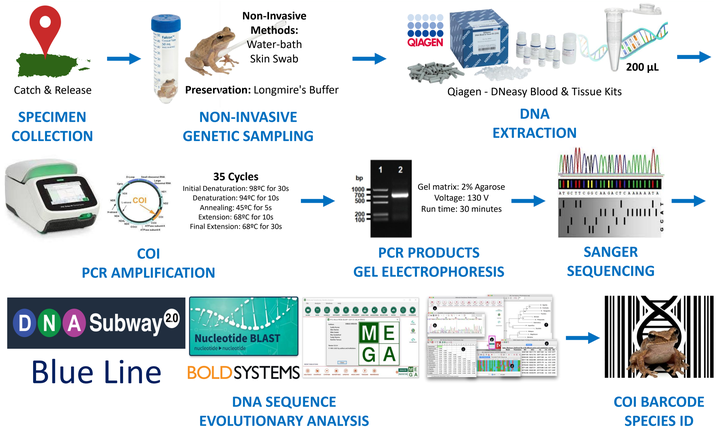
General Objective:

- Molecular identification of Puerto Rican amphibian tadpoles via COI gene DNA barcoding and non-invasive sampling, establishing a comparative framework for ecological research.

Specific Objectives:

- Collection of genetic material using strictly non-invasive methods (water baths and swabbing) to ensure animal welfare.
- Extraction, amplification, and sequencing of the COI gene to create reliable genetic reference barcodes for local tadpoles.
- Association of visually unidentifiable tadpoles with known adult species, overcoming morphological limitations.
- Establishment of a reproducible methodological protocol to strengthen genetic databases for future conservation efforts.

MATERIALS AND METHODS



COLLECTED INDIVIDUALS

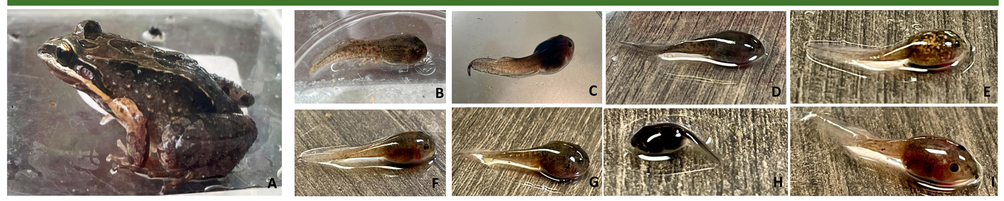


Figure. Amphibian specimens collected for this project. (A) Adult *Leptodactylus albilabris* (White-lipped frog). (B) Tadpole of the White-lipped frog. (C–I) A diversity of tadpoles belonging to various unidentified species. Sampling was conducted at a single pond in Cayey, Puerto Rico, where one *adult L. albilabris*, two L. *albilabris* tadpoles, and seven unidentified tadpoles were collected. All unknown specimens were obtained from the same aquatic habitat. All animal handling and sampling procedures were conducted under UPR-Río Piedras IACUC Protocol No. 2026-01-22 MBS.

SAMPLING METHODS

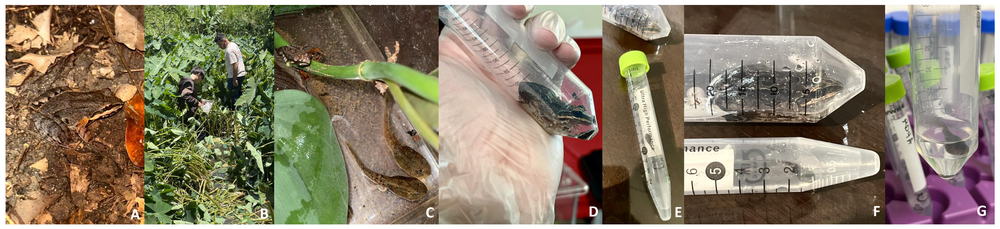


Figure. Sampling and non-invasive DNA collection procedures for *Leptodactylus albilabris*. (A) Adult *L. albilabris*. (B) Tadpole collection process in Cayey, Puerto Rico. (C) *L. albilabris* tadpoles and a developing froglet. (D–F) Adult and tadpole *L. albilabris* specimens undergoing the sterile water-bath sampling protocol of Brustenga et al. (2024) for the collection of environmental DNA, epithelial cells, and biological material. (G) An unidentified tadpole sampled using the same water-bath protocol. Although not shown, all adult frogs and tadpoles were also sampled using sterile nylon swabs as an alternative non-invasive method for collecting epithelial cells and tissue material for subsequent DNA extraction and molecular analyses.

PRELIMINARY RESULTS

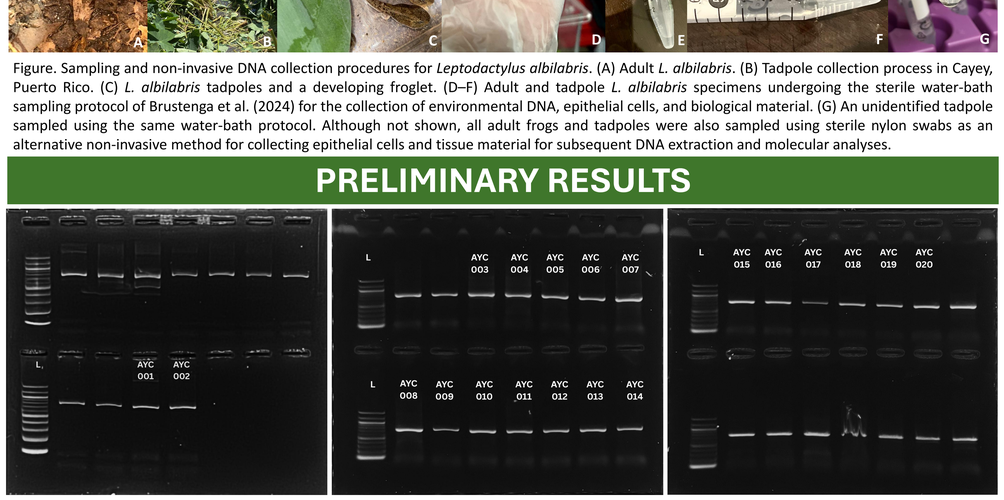


Figure. Agarose gel electrophoresis (2% agarose, 130 V for 30 minutes) of COI gene PCR amplification products. Lane L represents the 100 bp DNA ladder used as a molecular weight reference. Lanes labeled AYC 001 through AYC 020 demonstrate the successful amplification of the target mitochondrial cytochrome c oxidase subunit I (COI) gene. All labeled sample lanes exhibit a distinct, single band corresponding to the expected PCR product size of approximately 650 bp.

Table. DNA concentrations obtained from water-bath (WB) and swab (S) samples collected from *L. albilabris* and unidentified tadpoles in Cayey, Puerto Rico. DNA was quantified using a Thermo Scientific NanoDrop OneC spectrophotometer. Three measurements were performed per sample using 1 µL. Water-bath samples were collected according to the protocol of Brustenga et al. (2024), while swab samples were obtained using sterile nylon swabs.

		x̄ DNA Concentration (ng/µL)			
Species	**Stage**	**Sample Code**	**WB**	**Sample Code**	**S**
	Tadpole	AYC-001	8.9	AYC-002	5.0
L. albilabris	Adult	AYC-003	5.6	AYC-012	5.2
	Tadpole	AYC-004	4.3	AYC-013	6.1
	Tadpole	AYC-005	6.6	AYC-014	2.5
	Tadpole	AYC-006	7.1	AYC-015	5.5
	Tadpole	AYC-007	3.6	AYC-016	5.4
Unknown	Tadpole	AYC-008	7.0	AYC-017	7.8
	Tadpole	AYC-009	3.6	AYC-018	3.1
	Tadpole	AYC-010	7.7	AYC-019	6.6
	Tadpole	AYC-011	5.7	AYC-020	5.7
			x̄ 6.0		**x̄ 5.3**

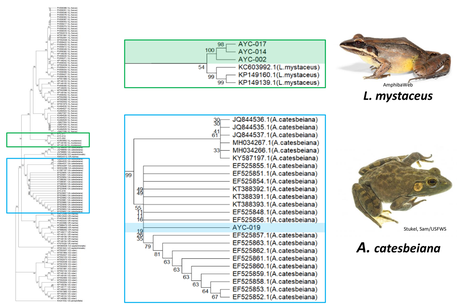


Figure. Neighbor-Joining phylogenetic tree based on 129 COI sequences, including samples generated in this study and reference sequences from GenBank. *Salamandrina perspicillata* (KX241532.1) was used as the outgroup. A, complete phylogenetic tree. B, samples AYC-002, AYC-014, and AYC-017 clustered with *Leptodactylus mystaceus* reference sequences with strong bootstrap support (98–100%). C, sample AYC-019 clustered within the American bullfrog clade, *A. catesbeiana* (formerly *Lithobates catesbeianus* and *Rana catesbeiana*). The tree was generated in MEGA12 using the Neighbor-Joining method with 10,000 bootstrap replicates.

DISCUSSION

- Both non-invasive sampling methods yielded measurable DNA concentrations and successfully amplified the target COI fragment (~650 bp), demonstrating that genetic material can be recovered from amphibian tadpoles without tissue removal or permanent harm to the organism (Brustenga et al., 2024; Gallardo et al., 2012).
- Although all samples produced PCR products of the expected size, only four generated sequences of sufficient quality for downstream analyses. Similar discrepancies between PCR amplification and sequencing success have been reported when non-target DNA is co-amplified in complex biological samples (Che et al., 2012; Leese et al., 2021).
- Several unsuccessful sequences matched non-target organisms, including bacteria (*Pseudomonas* spp.), insects, ticks, and other taxa. These results suggest co-amplification of environmental and non-target DNA, a known limitation of highly degenerate universal COI primers (Che et al., 2012; Leese et al., 2021).
- The CHMF4/CHMR4 primers were designed to maximize amplification across diverse amphibian lineages through the use of multiple degenerate bases (Che et al., 2012). While advantageous for DNA barcoding, this design may also increase amplification of non-target DNA when target DNA is scarce (Leese et al., 2021).
- PCR amplification was performed using KOD DNA polymerase, a high-fidelity enzyme optimized for specificity. In combination with highly degenerate primers and environmental samples, sequencing success may be influenced by interactions among primer design, DNA quality, and amplification efficiency (Che et al., 2012; Leese et al., 2021).
- DNA concentrations obtained from water-bath and swab samples were comparable (6.0 ng/µL and 5.3 ng/µL, respectively). However, validated COI sequences were recovered only from swab-derived samples. Because successful PCR amplification was obtained from both methods, additional studies are needed to better understand factors influencing sequencing success (Brustenga et al., 2024).
- Samples AYC-002, AYC-014, and AYC-017 clustered with *L. mystaceus* reference sequences. However, the large number of mismatches (~110) suggests these samples do not represent *L. mystaceus* but likely *L. albilabris*, the native Puerto Rican species, which lacks adequate COI representation in public databases (Rivero, 1978; Joglar, 1998).
- Sample AYC-019 clustered within the *A. catesbeiana* clade and differed from reference sequences by only 1–6 nucleotide positions, supporting its identification as American Bullfrog and demonstrating the utility of DNA barcoding for detecting introduced species during larval stages (Vences et al., 2005; Che et al., 2012).
- These results demonstrate the value of molecular approaches for resolving taxonomic uncertainty in amphibian tadpoles while highlighting the need to expand COI reference databases for Puerto Rican amphibians to improve future ecological and conservation studies (Ratnasingham & Hebert, 2013; Vences et al., 2005).

FUTURE WORK

- Expand sampling efforts to include additional tadpoles, species, and geographic regions throughout Puerto Rico.
- Evaluate alternative DNA polymerases, as KOD may be overly stringent for amplification with the highly degenerate amphibian COI primers CHMF4/CHMR4.
- Optimize PCR and sequencing protocols to reduce non-target amplification and improve recovery of amphibian-derived COI sequences.
- Generate reference COI barcodes for *L. albilabris* and other Puerto Rican amphibians currently underrepresented in public genetic databases.
- Compare the effectiveness of water-bath and swab sampling methods using larger sample sizes and multiple amphibian species.
- Develop a comprehensive DNA barcode reference library for Puerto Rican amphibians to support ecological monitoring, invasive species detection, and conservation efforts.

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