GENETIC CHARACTERIZATION OF THE CANE TOAD (*Rhinella marina*) IN PUERTO RICO USINGMITOCHONDRIAL COI BARCODES AND NON-INVASIVE SAMPLING METHODS

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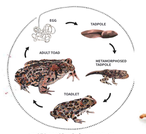
BACKGROUND

- The Cane Toad, *Rhinella marina*, is one of the most widely distributed invasive amphibians in the world and has successfully established populations throughout Puerto Rico since its introduction during the 1930s for agricultural pest control (Ross, 2007; Rivero, 1978).
- Due to its high reproductive output, ecological plasticity, and ability to thrive in disturbed habitats, *R. marina* has become an important model species for studies of biological invasions, adaptation, and population dynamics (Ross, 2007; Meshaka, 2001).
- The species undergoes a complex life cycle that includes aquatic larval (tadpole), juvenile, and adult stages, each exposed to different environmental conditions that may influence dispersal and population structure (Ross, 2007).
- Genetic studies of invasive species provide valuable information about introduction history, founder effects, population expansion, and potential adaptation to novel environments (Zhang et al., 2024; Everts et al., 2025).
- DNA barcoding based on the mitochondrial cytochrome c oxidase subunit I (COI) gene has become a widely accepted tool for species identification, biodiversity assessment, and genetic characterization of amphibians (Hebert et al., 2003; Che et al., 2012).
- Despite the widespread distribution of *R. marina* in Puerto Rico, genetic information from local populations remains limited, particularly across different developmental stages.

Cane toad native (blue) and introduced (red) distribution. LiquidGhoul / GNU



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RATIONALE

- *R. marina* is one of the most successful invasive amphibians worldwide and has established abundant populations throughout Puerto Rico. Despite its widespread distribution, genetic information from Puerto Rican populations remains limited (Ross, 2007; Rivero, 1978).
- Invasive species may experience founder effects, genetic drift, and local adaptation after introduction into novel environments, potentially leading to genetic changes over time (Zhang et al., 2024; Sales et al., 2021).
- Evaluating tadpoles, juveniles, and adults provides an opportunity to characterize the species across its life cycle while assessing the effectiveness of non-invasive genetic sampling methods in different developmental stages.
- Characterizing COI barcodes from Puerto Rican populations will generate baseline genetic data for future studies on invasion biology, population structure, and biodiversity monitoring (Hebert et al., 2003; Ratnasingham & Hebert, 2013).
- Non-invasive methods such as water baths, dermal swabs, and buccal swabs offer ethical alternatives for obtaining DNA while minimizing impacts on animal welfare (Pidancier et al., 2003; Gallardo et al., 2012; Brustenga et al., 2024).

OBJECTIVES

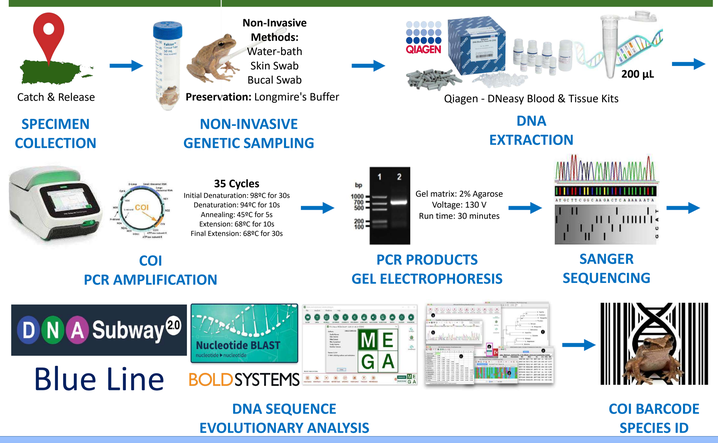
General Objective

- Genetically characterize tadpole, juvenile, and adult stages of the Cane Toad (*R. marina*) in Puerto Rico using mitochondrial COI DNA barcoding and non-invasive sampling methods.

Specific Objectives

- Obtain DNA from tadpoles, juveniles, and adults of *R.marina* using water-bath, dermal swab, and buccal swab sampling methods.
- Amplify and sequence the mitochondrial cytochrome c oxidase subunit I (COI) gene using DNA barcoding protocols.
- Evaluate the effectiveness of non-invasive sampling methods for recovering amphibian DNA across different developmental stages.
- Generate baseline genetic data for Puerto Rican populations of *R.marina* that may support future studies on invasion biology, population genetics, and biodiversity monitoring.

MATERIALS AND METHODS



COLLECTED SAMPLES

Figure. Collected *R. marina* specimens used in this study. (A–C) Adult Cane Toads collected in Cidra, Puerto Rico. (D–F) Juvenile individuals collected from the same locality. (G–I) Tadpoles representing the larval stage of R. marina. (J) Adult specimen maintained in the laboratory prior to non-invasive genetic sampling and molecular analyses. A total of three adults, three juveniles, and three tadpoles were included in the study. All animal handling and sampling procedures were conducted under UPR-Río Piedras IACUC Protocol No. 2026-01-22 MBS.

Sampling Methods

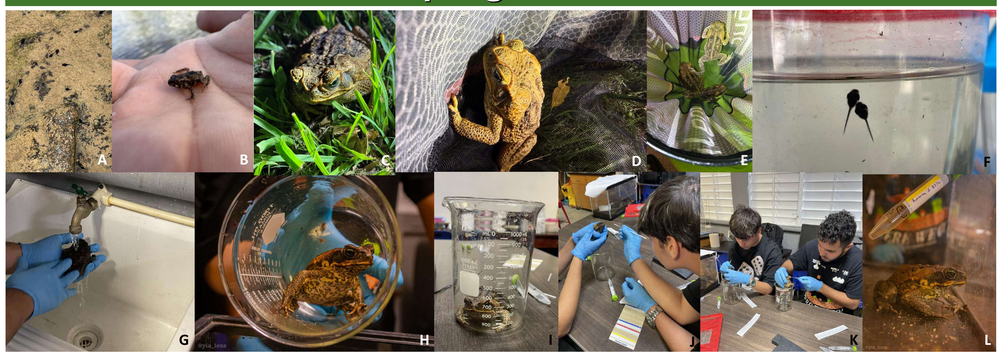


Figure. Field collection and non-invasive sampling procedures of *R. marina* in Cidra, Puerto Rico. (A) Tadpoles observed in a reservoir in Cidra. (B) Juvenile individual collected from the same locality. (C) Adult Cane Toad in its natural habitat. (D–E) Adult toads captured using fish nets. (F) Tadpoles maintained in small containers for transport and subsequent laboratory analyses. (G) Adult specimens being rinsed prior to sampling to remove excess soil and debris. (H) Adult toad prepared for water-bath sampling. (I) Water-bath procedure used to obtain environmental DNA from an adult individual. (J–K) Buccal and dermal swab sampling procedures. (L) Representative specimen following non-invasive sampling; collected samples were preserved in Longmire's buffer for subsequent DNA extraction and molecular analyses.

RESULTS

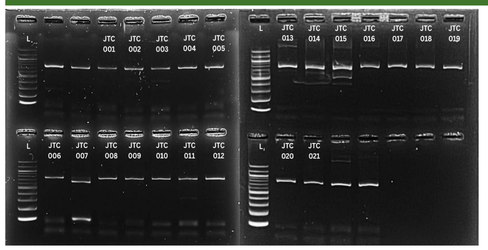


Figure. Agarose gel electrophoresis of COI gene PCR amplification products from R. marina samples. Lane L represents the 100 bp DNA ladder. Lanes JTC001–JTC021 correspond to tadpole, juvenile, and adult samples collected in Cidra, Puerto Rico. PCR products were separated on a 1.5% agarose gel and electrophoresed at 100 V for 45 min. Most samples exhibited a distinct band of approximately 650 bp, corresponding to the expected COI barcode fragment amplified with primers CHMF4 and CHMR4 (Che et al., 2012). Faint secondary bands observed in some samples likely represent non-specific amplification products, a common occurrence when using degenerate primers and non-invasive DNA samples.

Table. Mean DNA concentration (ng/µL) of *Rhinella marina* samples obtained through water-bath (WB), dermal swab (S), and buccal swab (B) methods. DNA concentration was measured using a NanoDrop OneC (Thermo Fisher Scientific). Values represent the mean of three readings obtained from 1 µL of DNA extract (estimated error ±2 ng/µL).

Species	Stage	Sample Code	x̄ DNA Concentration (ng/ WB	Sample Code	S	
R. marina	Tadpole	JTC-001	9.0	JTC-004	6.4	
		JTC-002	5.9	JTC-005	3.5	
		JTC-003	4.5	JTC-006	6.2	
	Juvenile	JTC-007	7.1	JTC-010	7.4	
		JTC-008	3.7	JTC-011	7.8	
		JTC-009	6.1	JTC-012	5.0	
	Adult	JTC-013	6.2	JTC-016	16.6	
		JTC-014	12.9	JTC-017	3.4	
		JTC-015	10.6	JTC-018	4.7	
			x̄ 7.3	x̄ 6.8	x̄	4.6

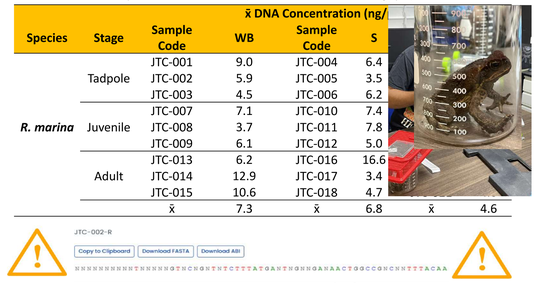


Figure. Representative Sanger sequencing result from a *R. marina* COI amplicon. Although all PCR products were submitted for sequencing, both sequencing attempts produced low-quality reads with numerous ambiguous bases (N), preventing sequence assembly and molecular identification.

DISCUSSION

- DNA was successfully recovered from tadpole, juvenile, and adult *R. marina* using water-bath, dermal swab, and buccal swab methods. All three approaches yielded measurable DNA concentrations, supporting the use of non-invasive sampling for amphibian genetic studies (Pidancier et al., 2003; Gallardo et al., 2012; Brustenga et al., 2024).
- PCR amplification produced the expected ~650 bp COI fragment in most samples, indicating successful amplification with the universal amphibian primers CHMF4 and CHMR4 (Che et al., 2012).
- Faint secondary bands observed in some samples suggest the presence of non-specific amplification products. Despite successful amplification, neither sequencing attempt generated COI sequences of sufficient quality for species identification or phylogenetic analyses. The first sequencing attempt used PCR products generated with KOD DNA polymerase and sequenced after an extended storage period, which may have affected sequence quality through DNA degradation or handling-related factors (Lorenz, 2012).
- A second sequencing attempt was performed using the original DNA extracts, which were amplified and processed by external collaborators using a different PCR protocol and polymerase. Because post-extraction procedures were conducted externally, the specific causes of sequencing failure could not be independently evaluated (Lorenz, 2012).
- The highly degenerate CHMF4 and CHMR4 primers were designed to maximize amplification across diverse amphibian taxa (Che et al., 2012). However, degenerate primers may also increase non-specific amplification and reduce sequencing quality when mixed or low-quality DNA templates are present (Che et al., 2012; Leese et al., 2021).
- Successful DNA recovery from tadpoles, juveniles, and adults demonstrates that non-invasive sampling can be applied across multiple life stages of *R. marina*, providing a useful framework for future amphibian genetic studies (Gallardo et al., 2012; Brustenga et al., 2024).
- Although usable COI sequences were not obtained, successful DNA extraction and amplification indicate that non-invasive methods remain promising for future molecular studies. Additional optimization of PCR product preservation, purification, transport, and sequencing workflows may improve sequencing success (Lorenz, 2012; Ratnasingham & Hebert, 2013; Brustenga et al., 2024).

FUTURE WORK

- Evaluate alternative DNA polymerases, as highly specific enzymes may not be optimal when using the degenerate amphibian COI primers CHMF4 and CHMR4 (Che et al., 2012).
- Implement additional PCR product purification and preservation strategies prior to sequencing to improve sequence quality and reduce potential degradation (Lorenz, 2012).
- Compare the effectiveness of water-bath, dermal swab, and buccal swab methods using larger sample sizes and multiple developmental stages (Gallardo et al., 2012; Brustenga et al., 2024).
- Expand sampling to additional *R. marina* populations throughout Puerto Rico to increase geographic representation.
- Generate COI barcode reference sequences for Puerto Rican populations to support future studies on invasion biology, population genetics, and biodiversity monitoring (Ratnasingham & Hebert, 2013).
- Compare Puerto Rican populations with introduced and native populations to investigate potential genetic changes associated with biological invasions (Zhang et al., 2024; Everts et al., 2025).

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