Genetic Characterization of the Puerto Rican Mountain Coquí (*Eleutherodactylus portoricensis*) and the Melodious Coquí (Eleutherodactylus wightmanae) Using the Mitochondrial COI Gene from Non-Invasive DNA Samples

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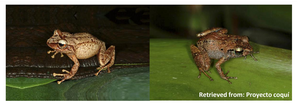
BACKGROUND



- Amphibians are vital as regulators of invertebrate populations, prey species, and bioindicators of environmental health, but in Puerto Rico their populations have suffered significant declines due to climate change, habitat loss, and environmental disturbances (Hedges et al., 2008; Joglar & Burrowes, 1996).
- The island is notable for its high level of endemism, especially within the genus *Eleutherodactylus*, which represents a major example of adaptive radiation in Caribbean amphibians (Joglar, 1998). The Mountain Coquí (*Eleutherodactylus portoricensis*) is an endemic species restricted to humid montane forests from Maricao to Luquillo, characterized by direct development (without a tadpole stage), and has shown population declines since the 1980s (Stewart & Woolbright, 1996; Joglar & Burrowes, 1996).
- The Melodious Coquí (*Eleutherodactylus wightmanae*) is another endemic species of Puerto Rico's humid montane forests, recognized by its distinctive call and preference for leaf litter and understory microhabitats (Rivero, 1978; Narins, 1983).
- Both species are highly vulnerable to climate change, microhabitat disturbances, and emerging diseases such as chytridiomycosis, which have contributed to amphibian population declines worldwide and in the Caribbean region (Hedges et al., 2008; Pabijan et al., 2012).

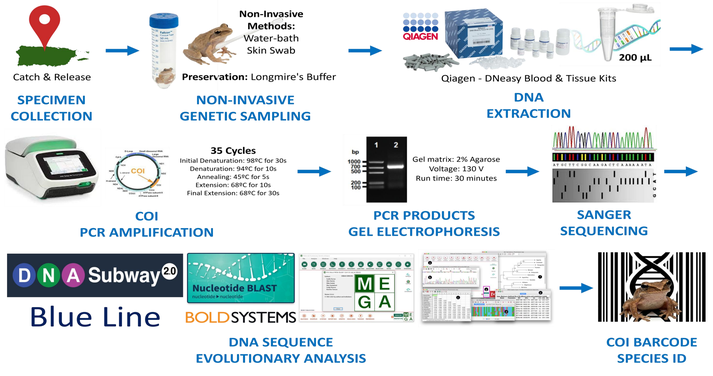
RATIONALE

- Amphibians are among the most sensitive vertebrate groups to environmental changes, making their study essential for understanding and protecting ecosystems (Hedges et al., 2008).
- In Puerto Rico, species such as the Mountain Coquí (*Eleutherodactylus portoricensis*) and the Melodious Coquí (*Eleutherodactylus wightmanae*) are key components of the island's biodiversity and ecological balance (Joglar, 1998). Despite their importance, there is still limited genetic information about these species, which restricts understanding of their diversity, population connectivity, and true level of vulnerability (Vences et al., 2005; Pabijan et al., 2012).
- This project proposes the use of DNA barcoding techniques (COI gene) to obtain reliable genetic data that will allow population comparison and support conservation strategies (Che et al., 2012; Ratnasingham & Hebert, 2013).
- Non-invasive methods align with ethical research principles by avoiding the removal of individuals from their natural habitats. Techniques like water baths allow sufficient DNA recovery without causing harm, prolonged stress, or mortality (Gallardo et al., 2012; Brustenga et al., 2024).
- Non-invasive sampling methods (such as buccal and dermal swabs) will also be used to avoid harm or stress to individuals (Gallardo et al., 2012; Martin et al., 2024).
- The research aims to expand genetic databases of Puerto Rican amphibians and establish an ethical, replicable protocol for future studies (Dufresnes & Jablonski, 2022). Overall, this project will contribute to the conservation and monitoring of unique species that are an essential part of Puerto Rico's natural identity (Hedges et al., 2008; Joglar & Burrowes, 1996).

OBJECTIVES

- Generate DNA barcodes of amphibians in Puerto Rico through sequencing of the mitochondrial COI gene.
- Integrate field-based morphological identification with molecular tools to enhance the taxonomic accuracy of *Eleutherodactylus* species.
- Apply non-invasive sampling methods (water bath and dermal swabs) to obtain DNA without causing harm to individuals.
- Investigate potential intraspecific genetic variation and population-level differences among amphibians across multiple locations in Puerto Rico.
- Develop a standardized and ethical protocol for the genetic characterization of amphibians in Puerto Rico.
- Enhance existing genetic databases of Puerto Rican amphibians to support future research in taxonomy, ecology, and conservation.
- Demonstrate the feasibility of incorporating non-invasive molecular methodologies into field-based amphibian research.

MATERIALS AND METHODS



COLLECTED INDIVIDUALS

Figure. Collected Individuals Representative amphibian individuals collected across multiple sampling sites in Puerto Rico are shown. Panels A–E correspond to *Eleutherodactylus wightmanae*, panels F–I to *Eleutherodactylus portoricensis*, and panels J–N to several unidentified *Eleutherodactylus* species (*Eleutherodactylus* sp.). Each specimen was morphologically identified in the field and handled using gloves to ensure biosecurity and minimize contamination. These individuals were subsequently used for non-invasive sampling and molecular analyses.

SAMPLING PROCEDURE

Figure. Sampling Procedure Non-invasive sampling methods used for DNA collection from amphibian individuals are illustrated. Each individual was sampled using both approaches to allow direct comparison between methods. Panel A shows the water bath method, used as the experimental group to collect environmental or skin-released DNA. Panel B depicts dermal swabbing performed under sterile conditions, representing the positive control group. Panel C shows the addition of Longmire's buffer for DNA preservation following sample collection. Panel D displays labeled sample tubes prepared for storage and subsequent molecular analyses. Panel E illustrates the quantification of extracted DNA using a NanoDrop spectrophotometer to assess DNA concentration and purity. All methods were designed to minimize handling stress and avoid harm to individuals while ensuring high-quality DNA collection. All procedures were conducted under approved protocol 2026-01-22 MBS from the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, Río Piedras Campus.

PRELIMINARY RESULTS

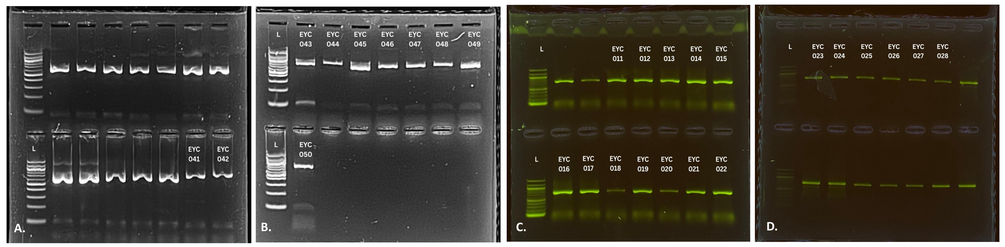


Figure. Amplification and Gel Electrophoresis Results Agarose gel electrophoresis images showing PCR amplification of the mitochondrial COI gene from amphibian samples are presented. Panels A–D display representative 2% agarose gels ran at 130 V for 30 minutes, with multiple lanes corresponding to individual samples and a 100 bp molecular weight ladder (L). Clear bands observed at the expected size of approximately 650 bp indicate successful DNA amplification across several specimens, while variation in band intensity reflects differences in DNA yield or amplification efficiency. PCR amplification and gel electrophoresis were performed by personnel at Arecibo C3. These results confirm the suitability of the extracted DNA for subsequent sequencing and molecular identification analyses.

Table. DNA Quantification Results DNA concentrations (ng/µL) varied across species and sampling methods. Swab (S) samples generally showed consistent or higher yields, while water bath (WB) samples exhibited greater variability. Detectable DNA was obtained for all species, indicating both methods are effective for collection.

	x̄ DNA Concentration (ng/µL)			
Species	**Sample Code**	**WB**	**Sample Code**	**S**
Eleutherodactylus sp.	EYC-041	5.3	EYC-046	8.1
	EYC-042	12.3	EYC-047	7.6
	EYC-043	7.2	EYC-048	8.5
	EYC-044	6.8	EYC-049	8.4
	EYC-045	9.5	EYC-050	6.7
E. portoricensis	EYC-0011	4.6	EYC-0015	2.8
	EYC-0012	3.7	EYC-0016	2.8
	EYC-0013	3.2	EYC-0017	2.9
	EYC-0014	4.9	EYC-0018	4.1
E. wightmanae	EYC-0019	5.0	EYC-0024	7.9
	EYC-0020	3.4	EYC-0025	7.4
	EYC-0021	6.4	EYC-0026	5.0
	EYC-0022	3.5	EYC-0027	1.6
	EYC-0023	3.0	EYC-0028	3.1
	x̄	5.6	x̄	5.5

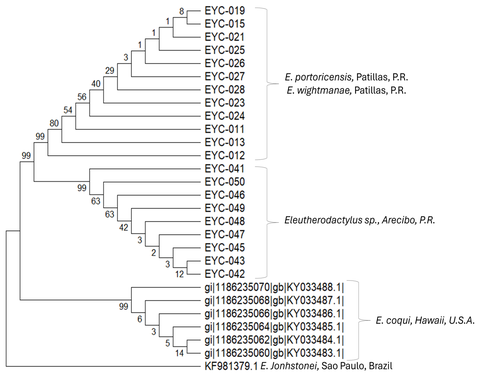


Figure. Phylogenetic Analysis Maximum Likelihood tree with bootstrap values (10,000 replicates) shown at nodes. The dataset included 28 sequences (674 bp) analyzed in MEGA12. Samples cluster by species, corresponding to *E. portoricensis*, *E. wightmanae*, and *Eleutherodactylus* sp., with reference sequences included for comparison.

DISCUSSION

DNA Yield and Amplification

- DNA concentrations (1.6–12.3 ng/µL) confirm successful extraction, but do not indicate DNA origin (Wilfinger et al., 1997).
- Clear ~650 bp bands suggest successful amplification, though fragment size alone does not confirm target specificity (Lorenz, 2012).

Amplification Bias (Polymerase + Primers)

- Use of KOD high-fidelity polymerase likely increased amplification bias toward templates with optimal primer matching, limiting recovery of low-abundance host DNA (McPherson & Møller, 2000).
- Degenerate primers (CHMF4/CHMR4) enhance taxonomic coverage but promote non-specific amplification in mixed DNA samples (Che et al., 2012).
- This may have favored non-target DNA while limiting consistent amplification of low-abundance host DNA, rather than completely preventing its amplification.

Non-Target Amplification

- BLAST results show dominant matches to:
  - *Pseudomonas fluorescens* (Patillas; ~45 bp mismatch)
  - *Rhodacarellus / Parasitidae* (Arecibo; ≥120 bp mismatch)
- Results indicate predominant amplification of non-target DNA, although host-derived sequences were successfully recovered in a subset of samples.

Host DNA Detection and Recovery

- A subset of samples (9/151) produced sequences correctly matching their respective species, confirming that host DNA was successfully recovered under current protocols.
- Successful identifications were obtained from both extraction methods (swab = 7; water bath = 2), indicating that non-invasive sampling can yield amplifiable host DNA.
- These results suggest that host DNA is present but inconsistently amplified, likely due to competition with more abundant microbial and environmental DNA.
- The low recovery rate (~6%) highlights limited amplification efficiency rather than complete methodological failure.

Microbiome and Template Competition

- Amphibian skin microbiota can exceed host DNA abundance, leading to template competition during PCR (McKenzie et al., 2012; Polz & Cavanaugh, 1998).
- Host DNA is likely present but in low relative abundance, resulting in preferential amplification of microbial templates.
- This imbalance reduces the recovery of target COI sequences despite successful DNA extraction and amplification.

Phylogenetic Signal vs Identity

- Despite non-target BLAST results, phylogenetic analysis shows structured clustering among samples.
- This pattern may reflect combined signals from low-abundance host DNA and shared environmental or microbial DNA composition.

Site-Specific Variation

- Differences between Patillas and Arecibo samples indicate location-dependent DNA composition, likely driven by local microbiota and environmental DNA inputs.

Implications

- COI amplification from dermal swabs is susceptible to non-target amplification but can still yield detectable host DNA under current protocols.
- Improving amplification specificity is necessary to increase the consistency and reliability of host identification.

FUTURE WORK

- Optimize PCR stringency by increasing annealing temperature (gradient PCR) to reduce non-specific primer binding and improve preferential amplification of host COI.
- Replace KOD polymerase with a more permissive Taq-based enzyme (e.g., OneTaq or Platinum Taq) to reduce amplification bias against low-abundance host DNA and improve recovery of true target sequences.
- Implement template dilution (1:10–1:20) to decrease the relative concentration of microbial DNA and mitigate template competition effects during PCR.
- Incorporate BSA into PCR reactions to improve amplification performance in the presence of residual inhibitors and stabilize enzyme activity in complex swab-derived samples.

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