Molecular Characterization of the COI Gene and DNA Barcode Generation in Four Common Coquí Species of Puerto Rico (*Eleutherodactylus coqui, E. antillensis, E. brittoni,* and *E. cochranae*) Using Non-Invasive DNA Extraction

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BACKGROUND

- Amphibians are highly vulnerable to environmental changes due to their permeable physiology and dependence on specific microclimatic conditions (Joglar & Burrowes, 1996; Stewart & Woolbright,1996).
- Population declines in Puerto Rican amphibians have been documented since the late 20th century, even among species once considered common or abundant (Joglar & Burrowes, 1996).
- The genus *Eleutherodactylus* represents the dominant component of Puerto Rico's terrestrial herpetofauna and serves as a key model for evolutionary, ecological, and conservation studies (Joglar,1998; Rivero, 1978).
- *Eleutherodactylus* includes 17 endemic species and is central to Puerto Rico's herpetofauna (Rivero, 1978; Joglar, 1998).
- Broad habitat range (forests to urban) reflects strong adaptive radiation (Schwartz & Henderson, 1991; Joglar, 1998).
- *E. coqui, E. antillensis, E. brittoni, and E. cochranae* coexist widely, enabling comparative studies (Rivero, 1978; Joglar, 1998).
- Species show ecological specialization (e.g., predator role, arid adaptation), but some remain poorly studied or cryptic (Drewry, 1970; van Berkum et al., 1982; Drewry & Rand, 1983; Schwartz & Henderson, 1991; Stewart & Woolbright, 1996).

RATIONALE

- Species such as *E. coqui, E. antillensis, E. brittoni, and E. cochranae* occupy diverse ecological niches, yet local-scale genetic data remain limited or nonexistent. (Joglar, 1998; Vences et al., 2005).
- Traditional identification methods (morphology and vocalizations) are limited by high external similarity, intraspecific phenotypic variation, and ecological overlap, potentially underestimating genetic diversity (Vences et al., 2005).
- The mitochondrial COI gene is a standardized tool for molecular identification and genetic comparison in amphibians, supporting conservation and environmental monitoring efforts (Che et al., 2012).
- Non-invasive sampling methods (dermal and buccal swabbing, and environmental DNA from water) enable DNA collection without significant harm, particularly important for small species (Pidancier et al., 2003; Gallardo et al., 2012; Brustenga et al., 2024; Martin et al., 2024).
- This study will strengthen genetic databases of Puerto Rican amphibians, evaluate concordance between traditional and molecular identification methods, and establish a replicable framework for future research in genetics, conservation, and biodiversity monitoring (Ratnasingham & Hebert, 2013; Vences et al., 2005).
- Traditional ID methods are limited for cryptic, juvenile, and sympatric taxa (Joglar, 1998).
- COI barcoding enables reliable taxonomy and genetic variation analysis (Che et al., 2012; Vences et al., 2005).
- Genetic diversity is key for conservation under land-use and climate changes (Joglar, 1998; Stewart & Woolbright, 1996).

OBJECTIVES

GENERAL OBJECTIVE

- To genetically characterize four representative species of Puerto Rican coqui frogs—*Eleutherodactylus coqui, E. antillensis, E. brittoni, and E. cochranae*—using DNA barcodes of the mitochondrial COI gene.

SPECIFIC OBJECTIVES

- Obtain genetic material from sampled individuals using non-invasive techniques the water bath method and dermal swabbing without compromising animal welfare.
- Amplify approximately 650-base-pair fragments of the COI gene via PCR for subsequent sequencing.
- Verify the taxonomic identity of the species through molecular analysis, evaluating the concordance between morphological and acoustic identification performed in the field and genetic evidence.
- Evaluate the feasibility and effectiveness of non-invasive molecular protocols applied to species of different body sizes and ecologies.
- Contribute to strengthening the genetic databases available for amphibians in Puerto Rico by generating sequences documented with photographic records, environmental variables, and morphological identification.

MATERIALS AND METHODS

COLLECTED INDIVIDUALS

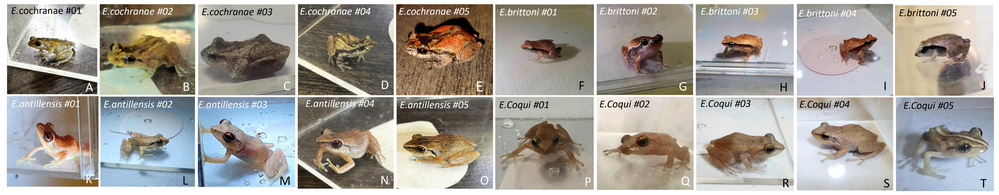


Figure. Collected Individuals Individuals are grouped by panels A–T, showing interspecific variation in morphology, size, and coloration among Puerto Rican coquí frogs. Panels A–E correspond to *Eleutherodactylus cochranae*, F–J to *Eleutherodactylus brittoni*, K–O to *Eleutherodactylus antillensis*, and P–T to *Eleutherodactylus coqui*. These individuals were used for non-invasive sampling and molecular analyses, with gloves worn during handling to prevent contamination of the specimens.

SAMPLING METHODS

Figure. Sampling Methods 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. 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

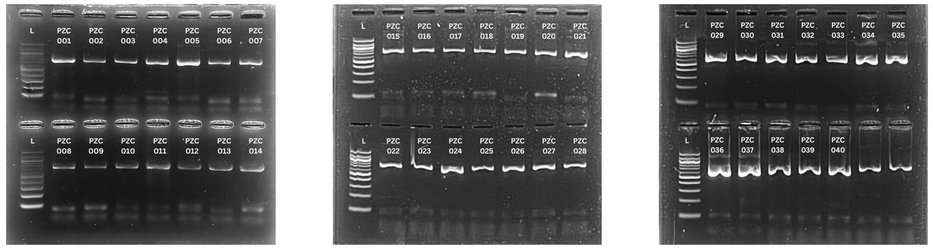
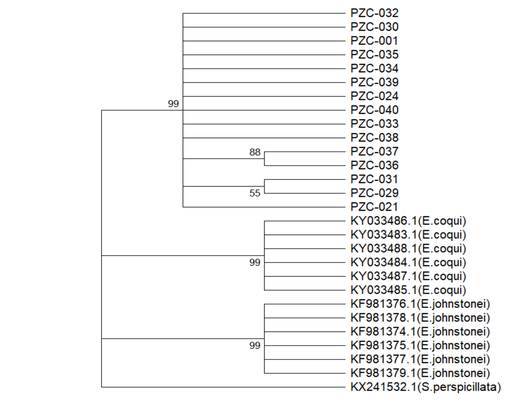


Figure. Amplification and Gel Electrophoresis Results Agarose gel electrophoresis images showing PCR amplification of the mitochondrial COI gene from amphibian samples are presented. Panels A–C display representative 2% agarose gels ran at 130 V for 30 minutes, with multiple lanes corresponding to individual 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. 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 among species and sampling methods. Water bath (WB) samples generally showed higher values and greater variability (mean = 11.5 ng/µL), with notably elevated concentrations in *E. cochranae* and *E. antillensis*. Swab (S) samples exhibited slightly lower but consistent yields across species (mean = 9.5 ng/µL). Detectable DNA was obtained from all samples, supporting successful extraction and suitability for downstream molecular analyses.

	x̄ DNA Concentration (ng/µL)			
Species	**Sample Code**	**WB**	**Sample Code**	**S**
E. cochronae	PZC-001	10.2	PZC-006	14.0
	PZC-002	12.1	PZC-007	15.5
	PZC-003	11.4	PZC-008	11.1
	PZC-004	44.8	PZC-009	11.4
	PZC-005	15.3	PZC-010	11.9
E.brittoni	PZC-011	7.1	PZC-016	3.2
	PZC-012	7.5	PZC-017	8.5
	PZC-013	7.4	PZC-018	11.4
	PZC-014	7.8	PZC-019	10.4
	PZC-015	7.7	PZC-020	11.7
E.coqui	PZC-021	6.9	PZC-026	5.6
	PZC-022	8.0	PZC-027	5.7
	PZC-023	9.6	PZC-028	3.9
	PZC-024	6.4	PZC-029	5.3
	PZC-025	6.6	PZC-030	4.7
E.antillensis	PZC-031	19.3	PZC-036	11.3
	PZC-032	10.6	PZC-037	12.0
	PZC-033	9.5	PZC-038	12.2
	PZC-034	10.6	PZC-039	11.2
	PZC-035	10.6	PZC-040	9.2
	x̄	11.5	x̄	9.5



Figuer. Evolutionary analysis by the Maximum Likelihood method The phylogenetic tree was inferred using the Maximum Likelihood method with the Tamura–Nei (1993) model. Bootstrap support values (10,000 replicates) are shown next to the branches, with values <50% collapsed. The analysis included 28 nucleotide sequences with 540 positions after partial deletion (90% site coverage). Rate variation among sites was modeled using a Gamma distribution (+G, parameter = 1.2875). Analyses were performed in MEGA12.

DISCUSSION

DNA Yield and Amplification

- DNA concentrations confirm successful extraction, but do not indicate DNA origin (Wilfinger et al., 1997).
- ~650 bp bands indicate successful PCR amplification, though not target specificity (Lorenz, 2012).

Amplification Bias (Polymerase + Primers)

- KOD polymerase likely increased bias toward optimal templates.
- Degenerate primers (CHMF4/CHMR4) promoted non-specific amplification in mixed DNA samples (Che et al., 2012).

Non-Target Amplification (Environmental DNA)

- BLAST matches to *Rhodacarellus*, *Parasitidae*, *Macrochelidae*, and *Leucomonia* showed high mismatches (~119–126 bp).
- Results indicate non-specific amplification of environmental eukaryotic DNA, not host COI.

Host DNA Recovery

- Some samples produced correct identifications, confirming host DNA presence.
- However, host DNA was underrepresented relative to environmental DNA, limiting recovery efficiency.

Environmental DNA and Template Competition

- Sampling methods capture microbiota and microfauna DNA from the environment.
- PCR favors abundant templates, leading to dominance of environmental DNA (Polz & Cavanaugh, 1998; McKenzie et al., 2012).

Post-Amplification Degradation Effects

- PCR was successful prior to shipment, but delayed sequencing likely caused partial amplicon degradation.
- Degradation reduces sequence quality and reliability (Sambrook & Russell, 2001; Tin et al., 2014).

Phylogenetic Signal vs Identity

- Tree topology shows structured clustering, suggesting partial biological signal.
- Patterns likely reflect mixed contributions of host and environmental DNA.

Site-Specific Environmental Signal

- Differences among samples reflect local environmental DNA composition, including soil-associated microfauna.

Implications

- Non-invasive COI amplification is affected by environmental DNA, PCR bias, and sample handling.
- Host DNA recovery is possible but requires optimization for reliable identification.

FUTURE WORK

- **Optimize PCR stringency (gradient PCR)** to reduce non-specific primer binding and improve selective amplification of host COI sequences.
- **Replace KOD polymerase with a more permissive Taq-based enzyme** to reduce amplification bias against low-abundance host DNA and improve target recovery.
- **Implement template dilution (1:10–1:20)** to decrease the relative concentration of environmental DNA and mitigate template competition effects.
- **Incorporate BSA into PCR reactions** to enhance amplification efficiency in complex, mixed DNA samples.
- **Evaluate shorter COI fragments (mini-barcodes)** to improve amplification success from degraded or low-quality DNA templates.
- **Apply post-PCR purification (e.g., bead cleanup or gel extraction)** to improve sequencing quality and reduce background noise.
- **Reduce time between amplification and sequencing** to minimize degradation of PCR amplicons and improve sequence reliability.
- **Assess environmental DNA contribution** (microbiota and microfauna) to better understand its impact on amplification outcomes.

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