Molecular Characterization of the COI Gene and DNA Barcode Generation in Three Historically Restricted Coquí Species of Puerto Rico (Eleutherodactylus jasperi, E. karlschmidti and E. eneidae) Using Collection Material

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BACKGROUND

- Lack of genetic data — Three species (*E. jasperi, E. eneidae, E. karlschmidti*) lack modern genetic data, relying mainly on morphology (Rivero, 1978; Burrowes et al., 2004).
- Unique reproduction — *E. jasperi* is the only ovoviviparous amphibian in the Americas (Rivero, 1978; Burrowes et al., 2004).
- Morphological traits of E. eneidae — *E. eneidae* is medium-sized, inhabits leaf litter, and shows dark reticulated eye venation (Rivero, 1959; Rivero, 1978; Burrowes et al., 2004).
- Traits of E. karlschmidti — *E. karlschmidti* is larger, with hind-limb webbing and a distinct diploid number; unseen since 1970 (Rivero et al., 1963; Rivero, 1978; Burrowes et al., 2004).
- Research limitations — Missing genetic characterization restricts scientific study and conservation planning (Rivero, 1978; Burrowes et al., 2004).
- DNA barcoding approach — This project sequences the mitochondrial COI gene for precise molecular identification (Che et al., 2012).
- Integrative taxonomy — Combining morphology with genetics helps resolve taxonomic uncertainty and supports conservation (Mayer & Lazell, 1988; Dufresnes & Jablonski, 2022).

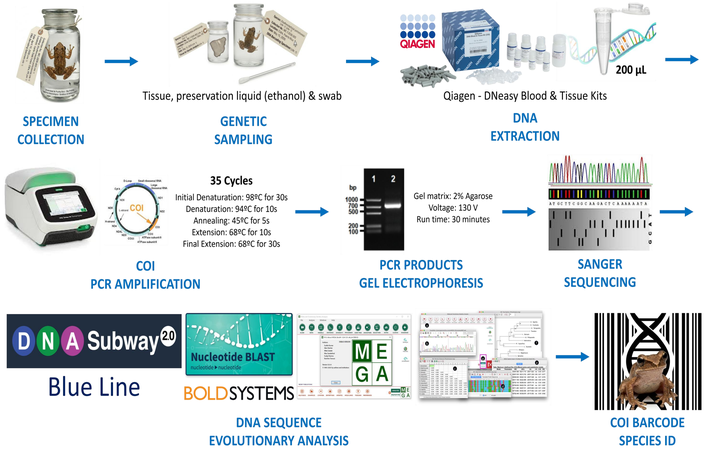
RATIONALE

- Amphibian vulnerability — Amphibians face severe global declines due to microhabitat dependence (Wake & Vredenburg, 2008).
- Loss of lineages — Species loss eliminates unique evolutionary lineages of high scientific value (Wake & Vredenburg, 2008).
- Lack of genetic data — *E. jasperi*, *E. eneidae*, and *E. karlschmidti* lack genetic or molecular data, relying only on historical descriptions (Vences et al., 2005).
- Museum specimens as DNA source — Preserved specimens are the only feasible DNA source for these critically endangered or possibly extinct species (Vences et al., 2005; Che et al., 2012).
- COI barcoding — The project uses mitochondrial COI barcoding to recover genetic information without affecting wild populations (Che et al., 2012).
- Chromosomal variation — Variation in diploid chromosome number suggests complex evolutionary histories requiring molecular confirmation (Bogart, 1981).
- Urgency of baseline data — Ecological vulnerability increases the urgency for baseline genetic data (Drewry, 1970; Rivero, 1978).
- Support for taxonomy — Molecular characterization will strengthen databases and aid future taxonomic revisions for the three species (Mayer & Lazell, 1988; Dufresnes & Jablonski, 2022).

OBJECTIVES

- Characterize COI gene — Characterize the mitochondrial cytochrome c oxidase subunit I (COI) gene in three coqui frog species endemic to Puerto Rico: *Eleutherodactylus jasperi*, *E. eneidae*, and *E. karlschmidti*, integrating historical morphological data with modern molecular tools.
- Conduct DNA barcoding — Perform DNA barcoding using preserved material from scientific collections to obtain genetic profiles of the three species.
- Generate genetic evidence — Produce reliable genetic evidence to clarify the taxonomic status, identity, and evolutionary lineage of these species, whose historical records rely solely on morphology.
- Strengthen databases — Contribute reference sequences to scientific databases, emphasizing the importance of museum collections for recovering information on rare, restricted-range, or potentially extinct species.
- Lay groundwork for future research — Establish a foundation for future studies on conservation, monitoring, taxonomy, evolution, and biodiversity of Puerto Rico's amphibians.

MATERIALS AND METHODS



SAMPLES

Figure Morphological views of a preserved amphibian specimen. Representative images showing dorsal, ventral, and lateral orientations, highlighting external anatomical features, limb structure, and translucent tissues. Although used for morphological reference and methodological standardization, this specimen does not correspond to any of the three target species originally selected for this study due to limitations in specimen availability.

SAMPLING PROCEDURE

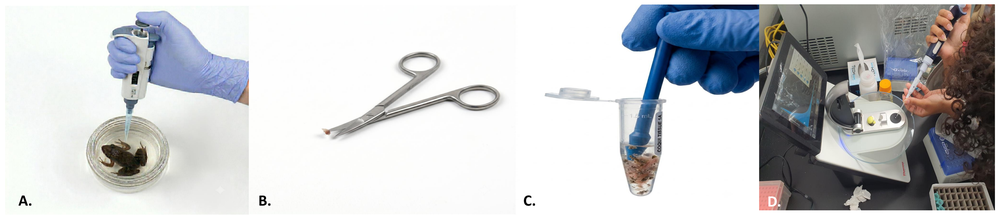


Figure. Sampling Procedure Overview of the sampling workflow for amphibian specimens. Panel A shows DNA extraction from preservation liquid (ethanol) containing biological material. Panel B displays sterile forceps used for handling tissue samples. Panel C illustrates the maceration of tissue samples prior to DNA extraction. Panel D shows DNA quantification using a NanoDrop spectrophotometer. 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. Agarose gel electrophoresis of PCR amplification products. DNA fragments were separated on a 2% agarose gel run at 130 V for 30 minutes, using a 100 bp ladder (L) as a molecular size marker. The expected amplicon size was approximately 650 bp. Clear bands near this size are observed across multiple samples (MTC 002–MTC 008), indicating successful amplification of the target fragment. Differences in band intensity suggest variation in DNA concentration or amplification efficiency among samples. PCR amplification and gel electrophoresis were performed by personnel at Arecibo C3.

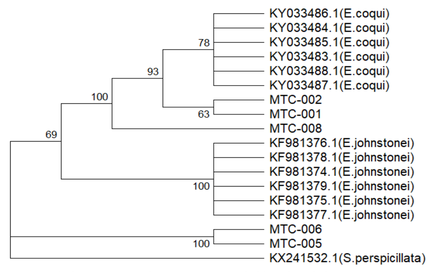


Figure. Phylogenetic Analysis Maximum Likelihood with bootstrap support values from 10,000 replicates shown at nodes (values <50% collapsed). The analysis included 18 nucleotide sequences with 507 positions after partial deletion (90% site coverage). Analyses were conducted in MEGA12. Samples cluster according to their evolutionary relationships, supporting species-level grouping.

Figure. DNA Quality and Quantification NanoDrop spectrophotometer output showing the absorbance spectrum and concentration values for a representative DNA sample (highlighted in blue). The peak at ~260 nm confirms the presence of nucleic acids, while A260/A280 and A260/A230 ratios indicate moderate DNA purity. The sample exhibited a concentration of 54.8 ng/µL, supporting successful extraction and suitability for downstream molecular analyses.

Table. DNA Quantification Results DNA concentrations (ng/µL) varied across sampling methods for the Unknown species. Tissue (T) samples showed the highest yield and variability (11.2–52.9 ng/µL), while preservation liquid (PL) and swab (S) samples exhibited lower and more consistent concentrations (PL: 5.4–15.0 ng/µL; S: 5.3–5.9 ng/µL). Detectable DNA was obtained across all methods, confirming successful extraction and suitability for downstream molecular analyses.

	x̄ DNA Concentration (ng/µL)					
Species	Sample Code	T	Sample Code	PL	Sample Code	S
Unknown	MTC-001	25.1	MTC-004	15.0	MTC-007	5.9
	MTC-002	11.2	MTC-005	5.7	MTC-008	5.3
	MTC-003	52.9	MTC-006	5.4		
	x̄	29.7	x̄	8.7	x̄	5.6

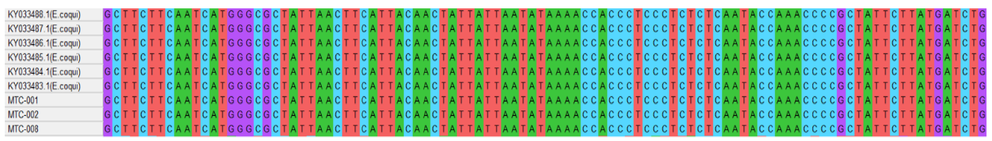


Figure. Sequence Alignment Multiple sequence alignment of partial COI gene sequences from amphibian samples (MTC-001, MTC-002, and MTC-008) and reference sequences of *E. coqui*. Conserved and variable nucleotide positions are displayed across aligned sequences, with color-coded bases indicating nucleotide differences. High sequence similarity between the sampled individuals and reference sequences supports species-level identification and confirms sequence quality for downstream phylogenetic analysis.

DISCUSSION

DNA Yield and Recovery Across Sample Types

- DNA was recovered from tissue (T), preservation liquid (PL), and swab (S), confirming feasibility from preserved specimens.
- Tissue showed the highest yield, while PL and S yielded lower but consistent DNA.

Amplification Success and DNA Integrity

- Amplification of ~650 bp COI fragments indicates sufficient DNA integrity despite degradation (Tin et al., 2014).
- Band variation reflects differences in DNA quality and template availability.

Host DNA Recovery (Method Validation)

- Sequences showed high similarity with *Eleutherodactylus*, confirming host DNA recovery.
- Despite absence of target species, results validate the methodological approach (Wandeler et al., 2007).

Influence of Preservation Medium

- PL contains dispersed and fragmented DNA, unlike tissue or swab.
- Lack of cellular protection reduces host DNA dominance in PCR, leading to weaker or non-specific matches.

Comparison of Sampling Approaches

- Tissue provides most reliable DNA due to higher integrity.
- Swabs offer a minimally invasive alternative with recoverable host DNA.
- PL is fully non-destructive but less efficient due to dilution and degradation.
- DNA recovery depends on cellular integrity vs dispersion in solution.

Phylogenetic Signal and Method Performance

- Tissue and swab clustered with reference *Eleutherodactylus*, supporting identification (Hebert et al., 2003).
- Reduced PL signal reflects fragmented and mixed DNA templates.

Methodological and Conservation Implications

- Preserved specimens are a valuable genetic resource, especially for rare or extinct species (Wandeler et al., 2007).
- Non-destructive methods allow DNA recovery while preserving specimens.

Limitations

- Absence of target species limits direct taxonomic conclusions.
- DNA degradation and variability affect consistency and amplification success.
- Nonetheless, results provide a validated framework for future studies.

FUTURE WORK

- Optimize PCR conditions (e.g., annealing temperature and cycle parameters) to improve amplification efficiency, particularly for low-quality DNA from preservation liquid samples.
- Evaluate shorter COI fragments (mini-barcodes) to improve amplification success from degraded DNA typical of preserved specimens.
- Improve DNA recovery from preservation liquid by concentrating samples or increasing input volume to enhance host DNA representation.
- Incorporate PCR additives (e.g., BSA) to improve amplification performance in samples with degraded or low-concentration DNA.
- Compare additional museum specimens to assess reproducibility and variability across preservation conditions and specimen age.
- Test alternative preservation conditions (e.g., ethanol vs isopropanol) to evaluate their effect on long-term DNA integrity.
- Expand reference datasets by generating validated sequences for *Eleutherodactylus* species, improving future taxonomic identification.
- Apply optimized protocols to target species (*E. jasperi, E. eneidae, E. karlschmidti*) when specimens become available.

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