ANALYSIS OF GENETIC AND MORPHOLOGICAL VARIABILITY OF THE COMMON COQUÍ (*Eleutherodactylus coqui*) IN PUERTO RICO USING THE MITOCHONDRIAL COI GENE AND NON-INVASIVE SAMPLING METHODS

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BACKGROUND

- The common coquí, *Eleutherodactylus coqui*, is one of Puerto Rico's most abundant and culturally significant amphibians, occupying a wide variety of habitats throughout the island (Rivero, 1978; Joglar, 1998).
- Previous studies have documented considerable variation in dorsal coloration, stripe patterns, bars, and spots among Puerto Rican populations of *E. coqui* (Woolbright & Stewart, 2008).
- Long-term monitoring identified at least 21 distinct dorsal pattern morphs, suggesting extensive phenotypic diversity within the species (Woolbright & Stewart, 2008).
- Morphological variation has been proposed to be influenced by habitat characteristics, predator-mediated selection, and local adaptation processes (Woolbright & Stewart, 2008).
- Molecular studies have revealed substantial genetic structuring among Puerto Rican populations, indicating a more complex evolutionary history than previously recognized (Velo-Antón et al., 2007).
- DNA barcoding using the mitochondrial cytochrome c oxidase subunit I (COI) gene provides a standardized tool for evaluating genetic variation and comparing populations (Hebert et al., 2003; Ratnasingham & Hebert, 2007).

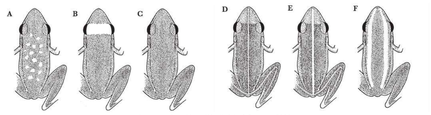


Image retrieved from Woolbright & Stewart, 2008

RATIONALE

- Morphological traits are frequently used for species identification and population characterization; however, phenotypic variation does not always reflect underlying genetic differentiation, making molecular validation necessary (Hebert et al., 2003; Vences et al., 2005).
- The existence of multiple dorsal pattern morphs in *E. coqui* raises the question of whether these visible differences represent simple phenotypic variation or are associated with genetic divergence among individuals and populations (Woolbright & Stewart, 2008).
- Understanding the relationship between morphology and genetic variation is important for interpreting patterns of population differentiation, local adaptation, and evolutionary processes within widely distributed species (Velo-Antón et al., 2007).
- Evaluating dorsal pattern variation using a standardized genetic marker such as the mitochondrial COI gene provides an opportunity to determine whether external morphological traits can serve as indicators of genetic diversity (Hebert et al., 2003; Ratnasingham & Hebert, 2007).
- Establishing a combined morphological and molecular dataset will provide a valuable reference for future studies involving amphibian biodiversity, population genetics, conservation, and DNA barcoding initiatives in Puerto Rico.

OBJECTIVES

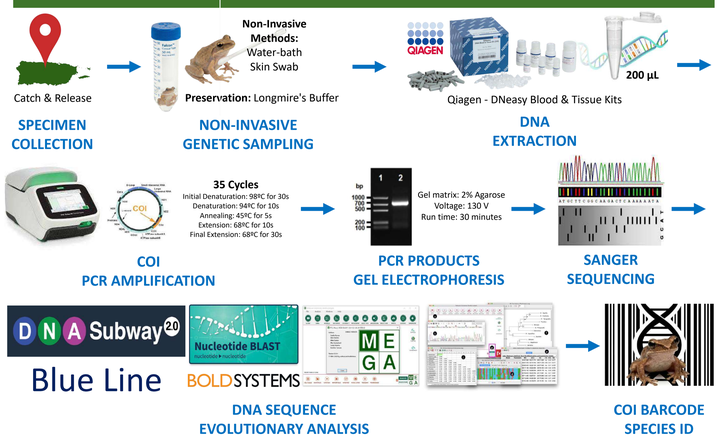
General Objective:

- Evaluate whether dorsal morphological variation in Puerto Rican populations of *E. coqui* is associated with genetic variation in the mitochondrial cytochrome c oxidase subunit I (COI) gene using non-invasive DNA sampling methods.

Specific Objectives:

- Document and classify dorsal morphological patterns of *E. coqui* from multiple localities in Puerto Rico using standardized photographic records.
- Obtain DNA from individual frogs using non-invasive sampling techniques, including the water-bath method and dermal swabbing.
- Amplify and sequence the mitochondrial COI gene from sampled individuals.
- Compare COI sequences among individuals exhibiting different dorsal morphological patterns.
- Assess whether dorsal pattern variation is associated with detectable genetic differences within Puerto Rican populations of *E. coqui*.
- Establish a combined morphological and molecular reference dataset for future studies of population genetics, biodiversity, and conservation of Puerto Rican amphibians.

MATERIALS AND METHODS



COLLECTED INDIVIDUALS

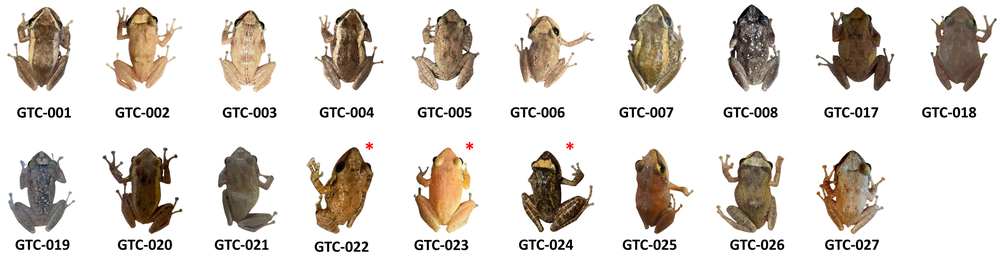


Fig. Phenotypic variation and dorsal polymorphism of the common coquí (*E. coqui*). The photographic composite displays the 19 collected specimens, highlighting significant plasticity in base coloration and dorsal patterns. These specimens represent individuals documented during field sampling across seven locations in Puerto Rico. Sampling sites encompass 3 locations in the municipality of Cidra, 2 in Patillas, 2 in Cayey, and 1 in Arecibo.

SAMPLING METHODS

Fig. Documentation of the field collection and non-invasive DNA sampling procedures used for *E. coqui* specimens. A, *E. coqui* individual observed within a bromeliad prior to collection. B, specimens transported in terrariums for temporary housing and processing. C, DNA sampling procedure. D, non-invasive water bath (WB) DNA collection following the protocol of Brustenga et al. (2024). E, skin swab DNA sampling. F, dorsal photograph obtained for documentation of color patterns and morphological characteristics. G, specimen photograph acquired for voucher documentation and individual record keeping prior to release. All animal handling and sampling procedures were conducted under UPR-Río Piedras IACUC Protocol No. 2026-01-22 MBS.

PRELIMINARY RESULTS

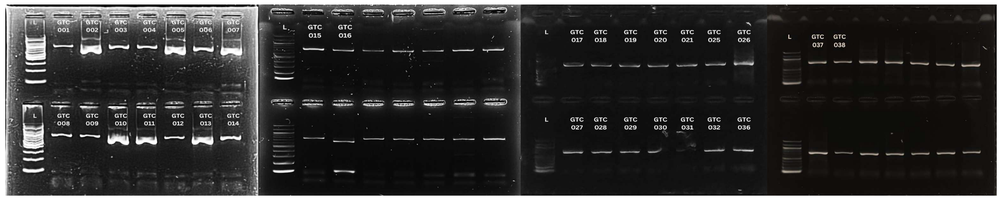


Figure. Agarose gel electrophoresis (2% agarose, 130 V for 30 min) of COI gene PCR amplification products generated using the primers Chmf4 and Chmr4 described by Che et al. (2012). Lane L represents the 100 bp DNA ladder used as a molecular weight reference. Lanes GTC001–GTC038 demonstrate product amplification. Most samples exhibited a distinct band corresponding to the expected PCR product size of approximately 650 bp. Variation in band intensity was observed among samples, and several lanes showed faint low-molecular-weight bands consistent with primer dimers or minor nonspecific amplification products; however, the target amplicon was clearly visible in all samples selected for downstream Sanger sequencing.

Table. Mean DNA concentration (ng/µL) obtained from *E.coqui* samples collected in Cayey, Cidra, Patillas, and Arecibo, Puerto Rico. DNA concentrations were measured using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific). Values represent the average of three independent readings obtained from 1 µL of DNA extract per measurement. Mean DNA concentrations were 6.1 ng/µL for WB samples and 6.8 ng/µL for swab samples. Results demonstrate that the Brustenga et al. (2024) water bath method yielded DNA concentrations comparable to those obtained through skin swabbing.

	x̄ DNA Concentration (ng/µL)			
Species	**Sample Code**	**WB**	**Sample Code**	**S**
E. coqui	GTC-001	4.9	GTC-009	7.2
	GTC-002	9.2	GTC-010	7.5
	GTC-003	5.9	GTC-011	8.4
	GTC-004	5.6	GTC-012	7.9
	GTC-005	7.0	GTC-013	9.7
	GTC-006	5.1	GTC-014	6.3
	GTC-007	6.9	GTC-015	6.3
	GTC-008	9.1	GTC-016	5.2
	GTC-17	3.8	GTC-28	5.4
	GTC-18	5.4	GTC-29	8.3
	GTC-19	7.3	GTC-30	4
	GTC-20	6.3	GTC-31	8.3
	GTC-21	3.6	GTC-32	4.4
	GTC-25	6.7	GTC-36	5.5
	GTC-26	6.8	GTC-37	5.6
	GTC-27	4.7	GTC-38	8.6
	x̄	**6.1**	**x̄**	**6.8**

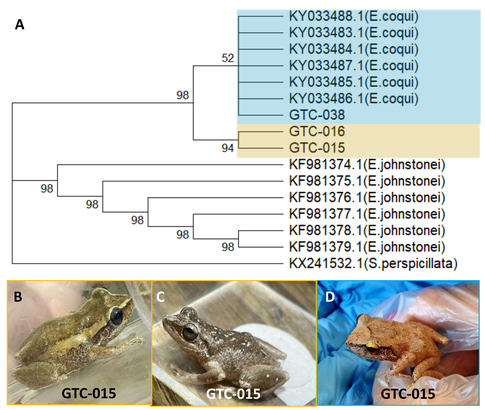


Figure. Neighbor-Joining phylogenetic tree based on COI sequences of *E. coqui* and GenBank reference sequences. *Salamandrina perspicillata* (KX241532.1) was used as the outgroup. All study samples clustered within the *E. coqui* clade. Samples GTC-015 and GTC-016 from Arecibo formed a distinct subclade, whereas GTC-038 from Patillas clustered with Hawaiian *E. coqui* reference sequences, suggesting geographic variation among populations. B–D, specimens used for COI sequencing and comparison with reference sequences. Tree 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, demonstrating that amphibian DNA can be recovered without tissue collection using minimally invasive approaches (Brustenga et al., 2024; Pidancier et al., 2003).
- Despite successful PCR amplification, only three swab-derived samples produced validated COI sequences, whereas no water-bath samples yielded sequences suitable for phylogenetic analysis in the present study. Similar discrepancies between PCR amplification and species detection have been reported when non-target DNA is co-amplified during COI-based analyses, particularly when target DNA occurs at low concentrations and environmental DNA is abundant (Che et al., 2012; Leese et al., 2021). The lack of validated water-bath sequences in this dataset does not necessarily indicate methodological failure, as successful DNA recovery using the same protocol has been achieved in other amphibian projects and species.
- Several unsuccessful sequences produced BLAST matches to non-target organisms, including *Pseudomonas* spp., arthropods (ticks), and other taxa. Amphibian skin hosts diverse microbial communities that may contribute DNA during non-invasive sampling procedures, potentially increasing the likelihood of non-target amplification (Herczeg et al., 2021).
- The recovery of non-target sequences is consistent with previous studies demonstrating that degenerate COI primers can amplify abundant environmental DNA from bacteria, fungi, algae, and other organisms when target DNA is present at low concentrations (Che et al., 2012; Leese et al., 2021).
- The extensive dorsal pattern variation observed among sampled individuals is consistent with previous reports describing substantial color-pattern polymorphism in Puerto Rican populations of *E. coqui* (Woolbright & Stewart, 2008).
- Phylogenetic analyses of the three validated sequences confirmed their placement within the *E. coqui* lineage. The clustering of Arecibo specimens into a distinct subclade and the association of one Patillas specimen with Hawaiian reference sequences are consistent with previous evidence of geographic genetic structuring among Puerto Rican populations (Velo-Antón et al., 2007).
- Because only three sequences were successfully validated, no definitive relationship between dorsal morphology and COI variation could be established. Nevertheless, the observed morphological diversity and preliminary genetic differences support continued investigation of the relationship between phenotypic and genetic variation in Puerto Rican populations of *E. coqui* (Woolbright & Stewart, 2008; Velo-Antón et al., 2007).

FUTURE WORK

- Optimize PCR and sequencing conditions to improve recovery of host-derived COI sequences and reduce non-target amplification.
- Evaluate alternative DNA polymerases, as KOD may be overly stringent for amplification with the highly degenerate amphibian COI primers CHMF4/CHMR4.
- Expand geographic sampling to include additional populations across Puerto Rico and increase the number of individuals representing different dorsal morphotypes.
- Further evaluate the performance of non-invasive sampling methods, including water-bath and skin swabbing techniques, across multiple amphibian species and environmental conditions.
- Investigate the relationship between dorsal pattern variation, geographic distribution, and genetic structure using larger datasets.
- Characterize amphibian-associated microbial communities to better understand their potential influence on DNA recovery and amplification success.

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ACKNOWLEDGEMENTS

Funded by the NIH (Grant No. 1 R25GM137212-01) and NSF Awards (ID 2321759, 2321760, and 2321761). We thank SEPA-UPR RP, UPR-Cayey, UPR-RP, Arecibo C3, Barcode Puerto Rico, and the Cold Spring Harbor Laboratory DNA Learning Center for their scientific collaboration, educational support, workshops, and contributions to STEM education and biodiversity research. Special thanks to Prof. Berríos from UPR-Cayey, Dr. Ríos from UPR-Ponce, Dr. Rodríguez, Dr. Borrero and Dr. Quesada from UPR Río Piedras; Dr. Ortiz, Dr. Fernández, Dr. Williams, Dr. Santiago from Arecibo C3, Mrs. Cecille Olmedo and to the school principal, Dr. Luis A. Otero for their guidance and support throughout the project. We also thank our mentor teachers, Charles A. Marzant Ortiz, M.Sc., and Edwin G. Ramírez Aponte, M.Sc.Ed., for their guidance and support. We thank our families, alumni, and community members for their participation and contributions to Misión Coquí, as well as the school communities of Benjamin Harrison Vocational High School (Cayey) and Ruth Evelyn Cruz Santos Vocational High School (Cidra).