Tracing the Source of the Non-Native Philippine Population of the Greenhouse Frog *Eleutherodactylus planirostris* (Cope, 1862) through DNA Barcodes

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ABSTRACT

Eleutherodactylus planirostris (Cope 1862), commonly called the Greenhouse Frog, is an insectivorous, direct-developing frog native to the Caribbean. It has been widely introduced outside of its native range and has been known to reach population densities of about 12,500 frogs per hectare, posing a potential ecological threat in areas of its introduction, especially to local insect populations. Recently, the species has been detected on several islands throughout the Philippines. Samples for this study were obtained from two locations in Quezon City (Luzon Island) and one location in Bacolod City (Negros Island). DNA barcoding using three genes (*Cytochrome b*, 16S rDNA, and *Cytochrome Oxidase subunit 1*) was performed with the objective of identifying the source population of Philippine *E. planirostris*. Our results indicate that *E. planirostris* samples in the Philippines are identical genetically to populations in Hawai'i and Florida, USA and are closely related to an individual from Matanzas, Cuba. A haplotype network built using the Fitch algorithm also supports the Cuban origin of the Philippine samples. Moreover, the Philippine specimens have nearly identical sequences for all three genes, which may have implications on its success as an introduced species.

Keywords: DNA Barcoding, *Eleutherodactylus planirostris, cytb*, 16S, *cox1*, Cuba

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INTRODUCTION

Eleutherodactylus planirostris, commonly known as the Greenhouse Frog, is a New World species native to a few islands in the Caribbean, specifically to Cuba, the Bahamas, and the Cayman Islands, a British Overseas Territory. The holotype described by Cope (1862) was collected in the island of New Providence in the Bahamas. The status of the Florida Keys population is unclear; genetic dating indicates that it is possible the Greenhouse Frog colonized the islands naturally when the Keys formed 125,000 years ago, but an artificial introduction from Matanzas, Cuba cannot be ruled out (Heinicke et al. 2011). It has been introduced to numerous areas (Figure 1), including mainland Florida and Louisiana (USA),

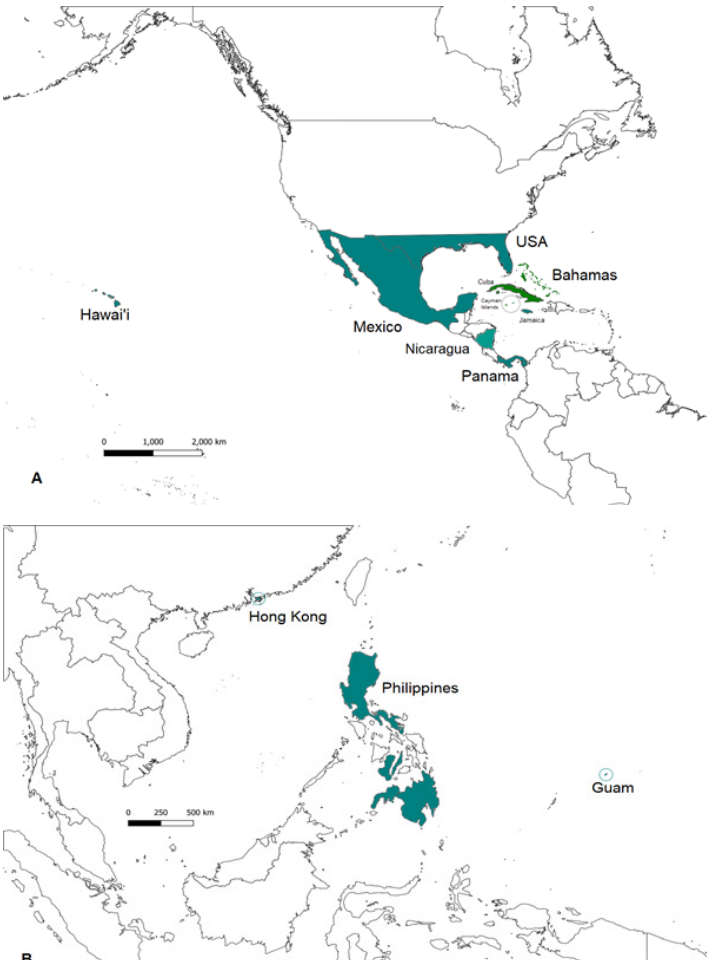

Figure 1. Distribution map of *Eleutherodactylus planirostris*. A: Native areas in the Caribbean (green) and areas of introduction in the Americas (blue). B: Areas of introduction in the Asia Pacific.

Nicaragua (Heinicke et al. 2011), Mexico (Cedeno-Vazquez et al. 2014), Panama (Crawford et al. 2011), Jamaica (Pough et al. 1977), Hawai'i (USA) (Krauss et al. 1999), and Guam (Christy et al. 2007). It belongs to the family Eleutherodactylidae, a family of terrestrial frogs that exhibit direct development of eggs, which are laid in moist soil, that hatch into froglets and skip the aquatic tadpole stage (Heinicke et al. 2007; Hedges et al. 2008). Adults are small, less than 30 mm Snout-Vent Length (SVL) (Olson et al. 2012a). It inhabits grasslands, shrublands, and non-aquatic subterranean habitats. Its highest altitudinal record is 727 meters above sea level (masl). Its diet consists primarily of leaf-litter invertebrates, predominantly ants, mites, and springtails (Olson and Beard 2012; Olson et al. 2012b). Since the species may reach high population densities (approximately 12,500 individuals per hectare in Hawai'i), they may constitute a potential threat to insect populations in their areas of introduction, particularly to ants (Olson and Beard, 2012; Olson et al. 2012a).

Two color variations of this species are known: a mottled tan and brown pattern, and a tan and brown pattern with two dorsolateral yellowish brown stripes (Lynn 1940). The striped pattern is apparently the dominant coloration in the frog's native range, with a ratio of three striped to one mottled frog being found in Cuba (Goin 1947). However, in areas outside of its native range, the mottled pattern becomes more common, with the introduced specimens in Guam all being of the mottled pattern and only 14% of the individuals caught in Hawai'i displaying the striped pattern (Goin 1947; Olson and Beard 2012; Olson et al. 2012a). Moreover, phylogenetic studies have found that two phylogenetically distinct groups exist within the species: one found in eastern Cuba, the Bahamas, and the Cayman Islands, and another in Western Cuba. The species is also not monophyletic: *Eleutherodactylus guanahacabibes*, a species endemic to the Guanahacabibes peninsula in Cuba, has been found to be nested within *E. planirostris* lineages based on analysis of *cytb* barcodes (Heinicke et al. 2011).

Recently, *E. planirostris* has also been detected in the Philippines, notably in Cebu (Sy and Salgo 2015), Luzon (Sy et al. 2015a), Mindanao (Olson et al. 2014), and Negros (Sy et al. 2015b), and is now present in eight islands in the archipelago (Pili et al. 2019) (see Figure 2 for pictures). The mode of introduction is probably through the ornamental plant trade (Christy et al. 2007; Olson et al. 2012a; Pili et al. 2019). Evidence for this comes from observations in Hawai'i and Guam, where the frogs are commonly first found around greenhouses and establishments that import plants, especially bromeliads (family Bromeliaceae) and *Dracaena* spp.

Figure 2. Photos of *E. planirostris* specimens found in the Philippines (not the ones used in this study). A: from Negros Occidental, Negros Island. B: from Barangay Diliman, Quezon City, Luzon Island. C: from Barangay Ugong Norte, Quezon City, Luzon Island. D: from Barangay Mariana, Quezon City, Luzon Island. Photos by Emerson Y. Sy.

(family Asparagaceae) (Krauss et al. 1999; Christy et al. 2007). *Eleutherodactylus planirostris* has been documented laying eggs in flowerpots (Goin 1944) and partially burying its eggs in moist earth (Iturriaga and Dugo-Cota 2018), and both adults and juveniles inhabit areas with moist earth (Goin 1947; Iturriaga and Dugo-Cota 2018), so introduction through the plant trade is not surprising. Records from the Bureau of Plant Industry (BPI) of the Department of Agriculture of the Philippine government indicate that ornamental plants were imported from the United States between 2005 and 2014 (e.g. *Tillandsia* sp., family Bromeliaceae, was imported in 2013), though no exact location in the US was given, and that palm seedlings were imported directly from Hawai'i in 2006. This time period coincides with the species' discovery in Davao in 2013 (Olson et al. 2014). In fact, many of the Philippine records of the species have been found in ornamental plant market stalls or in landscaped gardens featuring ornamental plants (Sy et al. 2015a; Olson et al. 2014) (see Figure 3).

Figure 3. An individual *E. planirostris* found in a potted bromeliad. Photo by Emerson Y. Sy.

Globally, the spread of Invasive Alien Species (IAS) has led to loss of biodiversity, damaged agriculture, and the introduction of both animal and human diseases (Early et al. 2016). Impacts on small island biodiversity are especially severe, since populations on these islands are small relative to continental populations (Russell et al. 2017; Simberloff 2000). The IUCN categorizes a species as Alien if it has become introduced to an area not within its natural past and present distribution; it becomes Invasive if it becomes problematic, such as by spreading disease or outcompeting native species, in its areas of introduction. Following this definition and given the lack of documented impacts on Philippine biodiversity, we do not yet advocate classifying the Greenhouse Frog as an Invasive Alien Species. Biological invasions of anurans have occurred before in the Philippines, such as the cane toad, *Rhinella marina* (Jabon et al. 2019; Pili et al. 2019). A study in the University of the Philippines Mindanao campus indicated that invasive frogs such as *R. marina* and *Kaloula pulchra* were the dominant anuran species on the campus, outcompeting native frog species and preying on local wildlife, mostly insects but also vertebrates such as the skink (Jabon et al. 2019). Further studies on the ecology of the Greenhouse Frog, especially its diet, are needed in order to inform an assessment of whether it should be considered an IAS. However, the potential negative impact of *E. planirostris* on local prey items, such as ants, is a cause for concern and studies are needed to document the local biology of *E. planirostris*, including its origin and mode of introduction. DNA barcoding, a method of characterizing species based on short, unique segments of their genomes, has proven useful in identifying newly discovered species, whether native or invasive (Blaxter 2003; Blaxter 2004; Crawford et al. 2011). In animals, the mitochondrial gene cytochrome oxidase subunit 1 (*cox1*) has become the recognized standard DNA barcode due to its ability to distinguish between related species across different lineages as well as detect possible cryptic species (Hebert et al. 2003; Hebert et al. 2004; Ward et

al. 2005). However, DNA barcoding relies on existing taxonomic databases founded on traditional morphological techniques; it is a method to discriminate between genetic lineages and cannot be used to replace taxonomy (Moritz and Cicero 2004; Hajibabei et al. 2017). Locally, barcoding of the native fish fauna in Taal Lake detected two new invasive species of cichlids while confirming the presence of five other invasive species, 15 native species, and one endemic species (*Sardinella tawilis*) (Aquilino et al. 2011). Moreover, it has also proven useful in tracing the origin of introduced species and in determining the presence of multiple introductions (Kolbe et al. 2004; Kolbe et al. 2008). It has already been utilized to trace the origins of invasive *E. planirostris* in Hawai'i, Panama, and Mexico using mitochondrial genes (Crawford et al. 2011; Heinicke et al. 2011; Cedeno-Vasquez et al. 2014). Crawford et al. (2011) used DNA barcoding of the mitochondrial large ribosomal subunit 16S to identify some specimens of *Eleutherodactylus* in Panama as *E. planirostris*. They conjectured that the Panamanian specimens came from Florida due to sequence identity. Heinicke et al. (2011) performed a similar study using the mitochondrial gene cytochrome b (*cytb*) for specimens in Florida, USA. Sequence identity indicates that Florida specimens originate from Matanzas, Cuba.

This study aimed to generate mitochondrial gene barcodes for Philippine specimens of *E. planirostris* and use these genes to trace the source population of this novel introduced species and to address the need for more *cox1* barcodes for amphibian taxa (Smith et al. 2008; Crawford et al. 2011).

MATERIALS AND METHODS

Acquisition of Samples

Eight samples in total were collected: seven from Quezon City, Luzon Island, and one from Bacolod City, Negros Island (Table 1). Morphometric data, namely Hind Limb Length (HLL), Tibia Length (TL), Snout-Vent Length (SVL), Snout Length (SL), Head Length (HL), Eye Diameter (ED), and Tympanic Diameter (TD), were measured for each specimen (Table 2). All specimens were deposited in the Natural History Museum of the Philippines, Manila City. *Eleutherodactylus planirostris* liver tissue samples were obtained from euthanized frogs and preserved in 99% ethyl alcohol by one of the authors (EYS). No local or national sampling permits were needed since sampling was done under the auspices of the Philippine National Museum (PNM), which is permitted to sample biodiversity. For similar reasons, Institutional Animal Care and Use Committee (IACUC) approval was not obtained due to the PNM's mandate to sample Philippine biodiversity.

Table 1. Collection data for specimens used in this study

Field Code	Collector	Locality	Collection		
			Date	Time	Method
EYS 387	John Martyr	Barangay Villamonte, Bacolod City, Negros Occidental, Negros Island; 10.67666° N, 122.96150° E	4 Sep 2014	08:00 – 17:00 H	Opportunistic Sampling
EYS 395	Emerson Y. Sy Benjamin Eleazar III	Barangay Mariana, Quezon City, NCR, Luzon Island; 14.618889° N, 121.033056° E	7 Aug 2014	08:00 – 17:00 H	Opportunistic Sampling
EYS 396	Emerson Y. Sy Benjamin Eleazar III	Barangay Mariana, Quezon City, NCR, Luzon Island; 14.618889° N, 121.033056° E	7 Aug 2014	08:00 – 17:00 H	Opportunistic Sampling
EYS 397	Emerson Y. Sy Benjamin Eleazar III	Barangay Mariana, Quezon City, NCR, Luzon Island; 14.618889° N, 121.033056° E	7 Aug 2014	08:00 – 17:00 H	Opportunistic Sampling
EYS 398	Emerson Y. Sy Benjamin Eleazar III	Barangay Mariana, Quezon City, NCR, Luzon Island; 14.618889° N, 121.033056° E	7 Aug 2014	08:00 – 17:00 H	Opportunistic Sampling
EYS 399	Emerson Y. Sy Benjamin Eleazar III	Barangay Mariana, Quezon City, NCR, Luzon Island; 14.618889° N, 121.033056° E	7 Aug 2014	08:00 – 17:00 H	Opportunistic Sampling
EYS 400	Emerson Y. Sy	Barangay UP-Diliman, Quezon City, NCR, Luzon Island; 14.65205° N, 121.04552° E	2 Oct 2014	08:00 – 17:00 H	Opportunistic Sampling
EYS 401	Emerson Y. Sy	Barangay UP-Diliman, Quezon City, NCR, Luzon Island; 14.65205° N, 121.04552° E	2 Oct 2014	08:00 – 17:00 H	Opportunistic Sampling

Table 2. Morphometric measurement of *E. planirostris* specimens

Specimen	HLL* (mm)	TL (mm)	SVL (mm)	SL (mm)	HL (mm)	ED (mm)	TD (mm)
EYS 387	27.15	11.35	22.75	3.75	8.75	3.05	1.95
EYS 395	35.25	11.00	20.50	4.30	8.75	3.10	1.35
EYS 396	36.85	11.55	23.85	4.30	9.65	3.55	1.80
EYS 397	34.30	10.60	20.40	4.00	8.55	2.70	1.85
EYS 398	27.85	8.40	17.00	3.55	7.45	2.60	1.30
EYS 399	29.25	9.00	17.40	3.65	7.85	2.50	1.70
EYS 400	32.75	10.10	19.05	3.80	7.80	2.35	1.65
EYS 401	26.45	7.80	15.00	2.70	6.30	2.35	1.25

*HLL=Hind Limb Length, TL= Tibia Length, SVL= Snout-Vent Length, SL= Snout Length, HL= Head Length, ED= Eye Diameter, TD= Tympanic Diameter.

DNA Extraction and Processing

DNA extraction was done via a modified form of the NaOH lysis or Alkaline lysis method (Bimboim and Doly 1979; as modified in Fontanilla et al. 2014), which relies upon manual and chemical disruption of the cell membrane to release the contents of the cytoplasm and membrane-bound organelles, including the mitochondria, and the separation of DNA and contaminants in iso-amyl alcohol and chloroform, respectively. DNA concentration and purity of extracts were measured using ThermoScientific™ NanoDrop 2000c Spectrophotometer.

DNA extracted from specimens of *E. planirostris* were then subjected to amplification via PCR. The primers for each gene are detailed in Table 3.

Table 3. Primers used for polymerase chain reaction (PCR) in this study

Gene	Primer Name and Sequence (5'-3')	Primer Size (bp)	Reference
Cytochrome B (*cytb*)	MVZ15L-mod (sense): AACTWATGGCCCMCACMATMCGWAA	1000	Moritz et al. 1992; as modified by Vences et al. 2003
	MVZ 16 (anti-sense): AAATAGGAAWTATCAWTCTGGTTTWAT		Moritz et al. 1992
16S	16S AR (sense): CGCCTGTTTATCAAAAACAT	650	Palumbi et al. 2002
	16S BR (anti-sense): CCGGTCTGAACTCAGATCACGT		
Cytochrome c Oxidase subunit 1 (*cox1*)	LCO 1490 (sense): GGTCAACAAATCATAAAGATATTGG	655	Folmer et al. 1994
	HCO 2198 (anti-sense): TAAACTTCAGGGTGACCAAAAAATCA		
	VF1 (sense): TTCTCAACCAACCACAARGAYATYGG	658	Ivanova et al. 2007
	VR1 (anti-sense): TAGACTTCTGGGTGGCCRAARAAYCA		

For all genes, a 25 µl PCR mix was prepared using the following components: 11.375µl of ultrapure water (Invitrogen™, Thermo Fisher Scientific, USA), 5 µl of Q Solution (Qiagen®, Germany), 2.5 µl of 10X PCR Buffer (Vivantis, Malaysia or

Promega, USA), 1.25 µl each of the appropriate forward and reverse primer (10 µM), 1.0 µl of 25 mM $MgCl_2$ (Roche, Switzerland), 0.5 µl of 10 mM dNTP (Invitrogen™, Thermo Fisher Scientific, USA), 0.125 µl of Taq DNA Polymerase (5 U/µl, Vivantis, USA or Promega GoTaq® Flexi DNA Polymerase 5U/µl, Promega, USA), and 2 µl of DNA template.

The conditions for the PCR run differed for each gene. For *cytb* (using MVZ15L-mod and MVZ 16), the initial denaturation was at 94 °C for 5 minutes, followed by 38 cycles at 94 °C for 30 seconds, 41 °C for 30 seconds, 72 °C for 60 seconds, and a final extension at 72 °C for 7 minutes (modified from Heinicke et al. 2011). For 16S (using 16AR and 16SBR) and *cox1* (using VF1 and VR1), a two-stage procedure was followed. The first stage required an initial denaturation at 94 °C for 5 minutes, followed by 5 cycles at 94 °C for 30 seconds, 45 °C for 30 seconds, and 72 °C for 45 seconds. The second stage involved 35 cycles at 94 °C for 30 seconds, 51 °C for 30 seconds, and 65 °C for 45 seconds, followed by a final extension step at 72 °C for 5 minutes (modified from Ivanova et al. 2007). For *cox1* (using HCO and LCO primers), the initial denaturation step was at 94 °C for 5 minutes, followed by 36 cycles at 94 °C for 30 seconds, 45 °C for 30 seconds and 65 °C for 1 minute, followed by a final extension step at 72 °C for 5 minutes (Folmer et al. 1992).

PCR was performed using either a MultiGene Optimax™ thermocycler (Labnet Inc., USA) or a TGradient thermocycler (Biometra®, Germany), depending on machine availability.

The PCR products were run through a 1% agarose gel stained with 1% Ethidium Bromide for 30 minutes and visualized under a UV Transilluminator. Kapa™ Universal Ladder was loaded alongside DNA samples in order to measure band molecular weight.

Gel extraction followed the QIAquick® Gel Extraction Kit by QIAGEN® protocol. To ensure the presence of DNA, 2 µl of extract was run again through agarose gel electrophoresis. DNA concentration of gel extracts was likewise measured using Thermo Scientific™ NanoDrop 2000c Spectrophotometer.

Sanger sequencing was performed by 1st BASE in Singapore. The purified DNA product was packaged together with 10 µl of the primers used during PCR.

Phylogenetic Analysis and Sequence Comparison

The sequences obtained from 1st BASE were checked and assembled using Pregap4 and Gap4 (Bonfield et al. 1995) of the Staden package (v. 2.0.0b10) (Staden et al.

2000). BLASTN (Nucleotide Basic Local Alignment Search Tool) (Altschul et al. 1990) was used to find the closest matches in GenBank (www.ncbi.nlm.nih.gov/genbank/) (Sayers et al. 2019). The sequences obtained for all three genes for each specimen were registered with the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). GenBank accession numbers for each gene from each specimen are given in Table 4.

Table 4. GenBank Accession numbers for each gene sequenced of each specimen

Specimen Field Code	GenBank Accession Number		
	cytb	16S	cox1
EYS 387	KT151767	KT151783	KT151775
EYS 395	KT151770	KT151786	KT151777
EYS 396	KT151771	KT151787	KT151778
EYS 397	KT151772	KT151788	KT151779
EYS 398	KT151773	KT151789	KT151780
EYS 399	KT151774	KT151790	KT151782
EYS 400	KT151768	KT151784	KT151781
EYS 401	KT151769	KT151785	KT151776

Greenhouse Frog sequences in GenBank were downloaded along with outgroup species and aligned alongside the query sequences using the ClustalW function (Thompson et al. 1994) in BioEdit (v. 7.0.9) (Hall 1999). To achieve uniform sequence length, longer sequences were trimmed to match the size of the shortest sequences. The outgroup chosen for 16S and *cox1* was *E. johnstonei* since sequences for this species for both genes were available in GenBank and it belongs to a separate subgenus (*Eleutherodactylus*) than *E. planirostris* (*Euhyas*) (Hedges et al. 2008) and branches clearly from *E. planirostris* and its sister taxa in the analysis of Pyron and Weins (2011). For *cytb*, *E. inoptatus* was used as outgroup based on the study by Heinicke et al. (2011) since it also belongs to another subgenus (*Pelorius*) (Hedges et al. 2008; Heinicke et al. 2011). Saturation testing was performed using the Xia Test (Xia et al. 2003; Xia and Lemey 2009) in DAMBE (v. 6.4.81) (Xia 2015; Xia 2017). Model testing was then performed for each gene using jModel Test 2 (v. 2.1.4) (Darriba et al. 2012), and the corrected Akaike Information Criterion score (Akaike 1973; Akaike 1974; Hurvich and Tsai 1993) to select the best fit model for phylogenetic analysis. Tree construction was done using the Neighbor-Joining (NJ) tree construction method (Saitou and Nei 1987) with 1000 bootstrap pseudoreplicates calculated for branch supports (Felsenstein 1985) using PAUP* (Swofford 2003), following the optimum models determined by jModel Test.

Separate trees were constructed for *cytb*, 16S and *cox1*. Neighbor-Joining (NJ) trees were constructed separately for each gene using the Kimura 3-Parameter model Gamma Correction (TPM1μf+G) (Kimura 1981) for the *cytb* alignment (666 bp), Generalized Time Reversible model (Tavare 1986) with Gamma correction (GTR+G) for the 16S alignment (464bp, including gapped sites), and the Hasegawa, Kishino, and Yano model (Hasegawa et al. 1985) with Gamma correction (HKY+G) for the *cox1* alignment (611 bp). For further analysis, a Maximum Likelihood (ML) tree was constructed for *cytb* sequences using IQ-TREE with 1000 Ultrafast Bootstraps (UFB) calculated as branch support (Minh et al. 2013; Nguyen et al. 2014).

The haplotype network was created using Fitchi (Matschiner 2015), a python script based on the Fitch algorithm (Fitch 1970). Statistical Dispersal-Vicariance Analysis (S-DIVA) was performed using RASP v 4.2 (Yu et al. 2015; Yu et al. 2020).

RESULTS AND DISCUSSION

BLAST Search Results

Nucleotide BLAST (BLASTN) search results showed that all the eight *cytb* sequences generated in this study matched with *Eleutherodactlyus planirostris* sequence in GenBank (accession no. HQ831590) with 100% sequence identity. All eight *cox1* sequences from this study had 100% sequence identity with *E. planirostris* in GenBank with accession no. JF69001. The eight 16S sequences from this study matched with *E. planirostris* GenBank accession nos. DQ283107 and KM252680 with sequence identity of 99.8% to 100%.

According to Heinicke et al. (2011), the largest mean intraspecific distance for *E. planirostris* using *cytb* is 7.3%, between Eastern and Western lineages of the species while interspecific distances calculated for other *Eleutherodactlyus* species revealed an uncorrected pairwise distance of greater than 12% (Rodriguez et al. 2013). Given the low percentage difference of our sequences from *E. planirostris cytb* sequences in GenBank, we conclude that our specimens are *E. planirostris*. Our identification is supported by 16S and *cox1* sequence data, which have a 99.8% to 100% sequence identity with *E. planirostris* sequences in GenBank.

Phylogenetic Trees

Both the NJ and ML trees constructed from *cytb* sequences (Figures 4 and 5) show that the Philippine population of *E. planirostris* groups with specimens from Hawai'i, Florida (including the Florida Keys), and one specimen from Carbonera, Matanzas, Cuba.

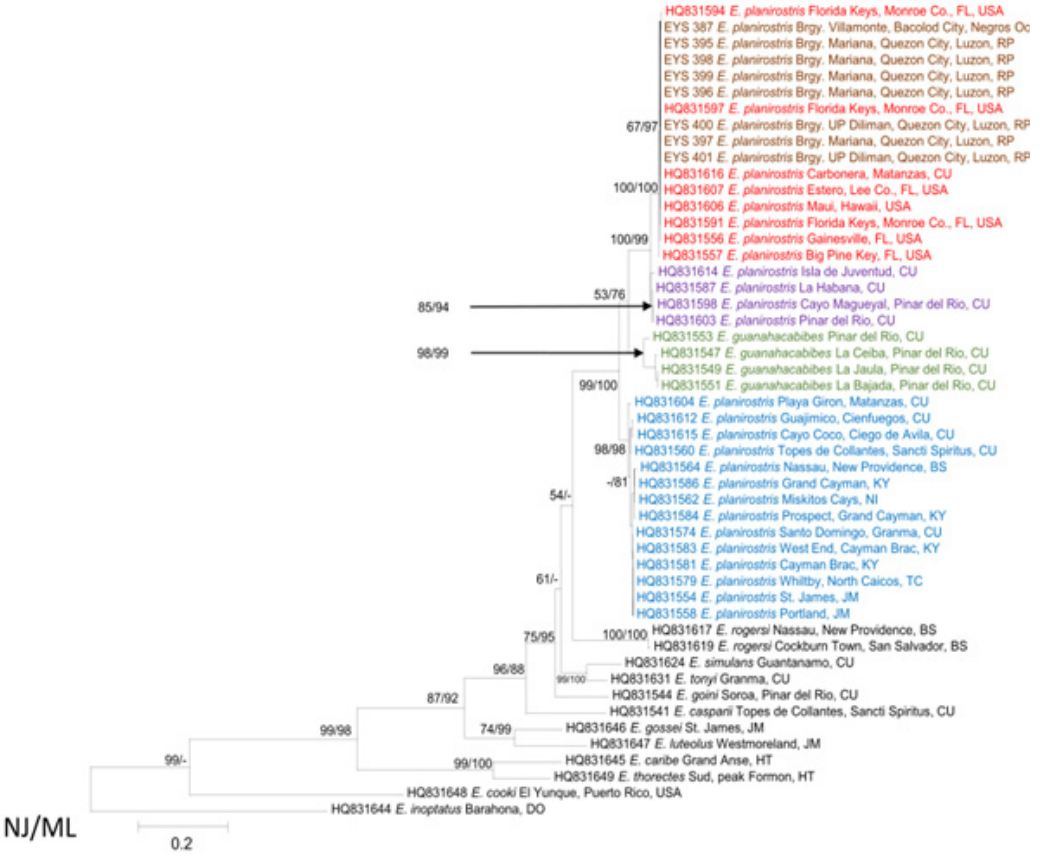

Figure 4. Phylogenetic tree of *E. planirostris* and related species using 666 bp *cytb* barcodes and the TPM1uf+G model of molecular evolution. Specimens whose labels start with EYS were obtained for this study while the remaining sequences were obtained from GenBank. The NJ tree is rooted on *E. inoptatus* based on the analysis of Heinick et al. (2011). Branches with 50% or greater bootstrap support (NJ/ML) have the corresponding number of bootstraps placed on the node. The country of origin of each sequence is placed beside the species name. Blue represents eastern *E. planirostris*, red and purple represent the Matanzas lineage and other lineages of western *E. planirostris*, respectively, brown corresponds to Philippine specimens of *E. planirostris* and green represents *E. guanahacabibes*. (CU=Cuba, KY= Cayman Islands, JM=Jamaica, BS=Bahamas, HT=Haiti, NI=Nicaragua, DO=Dominican Republic, RP=Philippines). The scale bar represents two nucleotide substitutions for every 10 nucleotides.

Figure 5. Subtree of the *cytb* tree (Figure 4) showing only *E. planirostris* sequences. Blue represents eastern *E. planirostris*, red and purple represent the Matanzas lineage and other lineages of western *E. planirostris*, respectively, brown corresponds to Philippine specimens of *E. planirostris,* and green represents *E. guanahacabibes*. Philippine specimens of *E. planirostris* group with the Matanzas lineage of Western *E. planirostris* and *E. guanahacabibes* groups with Western *E. planirostris*. Branches with 50% or greater bootstrap support (NJ/ML) have the corresponding number of bootstraps placed on the node. (CU=Cuba, KY= Cayman Islands, JM=Jamaica, BS=Bahamas, HT=Haiti, NI=Nicaragua, DO=Dominican Republic, RP=Philippines). The scale bar represents one nucleotide substitution for every 100 nucleotides.

These sequences represent the Matanzas lineage of Western *E. planirostris* (Heinicke et al. 2011), showing that the ultimate source of the Philippine population is Matanzas, Cuba and that it shares a common origin with the Hawai'i and Florida populations. The immediate source of the Philippine population is probably Florida or Hawai'i. This conclusion is supported by the 16S (Figure 6) and *cox1* (Figure 7) NJ trees, which show the Philippine population grouping with those from Florida and Panama City. The same is true of the haplotype network (Figure 8), which shows

Figure 6. Neighbor-joining tree of *E. planirostris* and related species using a 464 bp alignment (including gaps) of 16S barcodes and the GTR+G model of molecular evolution. Specimens whose labels start with EYS were obtained for this study while the remaining sequences were obtained from GenBank. The tree is rooted on *E. johnstonei* based on the analysis of Pyron and Weins (2011). Branches with 50% or greater bootstrap support have the corresponding number of bootstraps placed on the node. The country of origin of each sequence is placed beside the species name. Blue represents eastern *E. planirostris*, red and purple represent the Matanzas lineage and other lineages of western *E. planirostris*, respectively, brown corresponds to Philippine specimens of *E. planirostris,* and green represents *E. guanahacabibes*. (CU=Cuba, BS=Bahamas, PA=Panama, HK=Hong Kong, RP=Philippines). The scale bar represents five nucleotide substitutions for every 100 nucleotides.

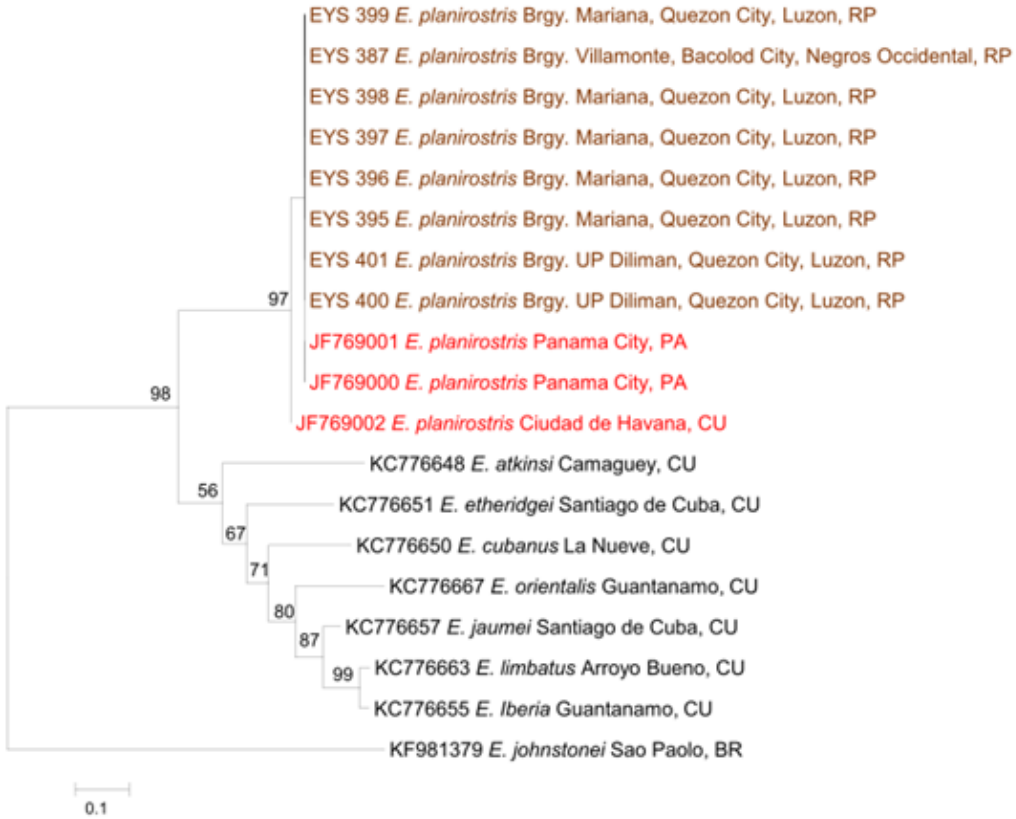

Figure 7. Neighbor-joining tree of *E. planirostris* and related species using 611 bp *cox1* barcodes and the HKY+G model of molecular evolution. Specimens whose labels start with EYS were obtained for this study while the remaining sequences were obtained from GenBank. The tree is rooted on *E. johnstonei* based on the analysis of Pyron and Weins (2011). Branches with 50% or greater bootstrap support have the corresponding number of bootstraps placed on the node. The country of origin of each sequence is placed beside the species name. Specimens in red represent western *E. planirostris*, while brown corresponds to Philippine specimens of *E. planirostris*. (CU=Cuba, PA=Panama, BR=Brazil, RP=Philippines). The scale bar represents one nucleotide substitution for every 10 nucleotides.

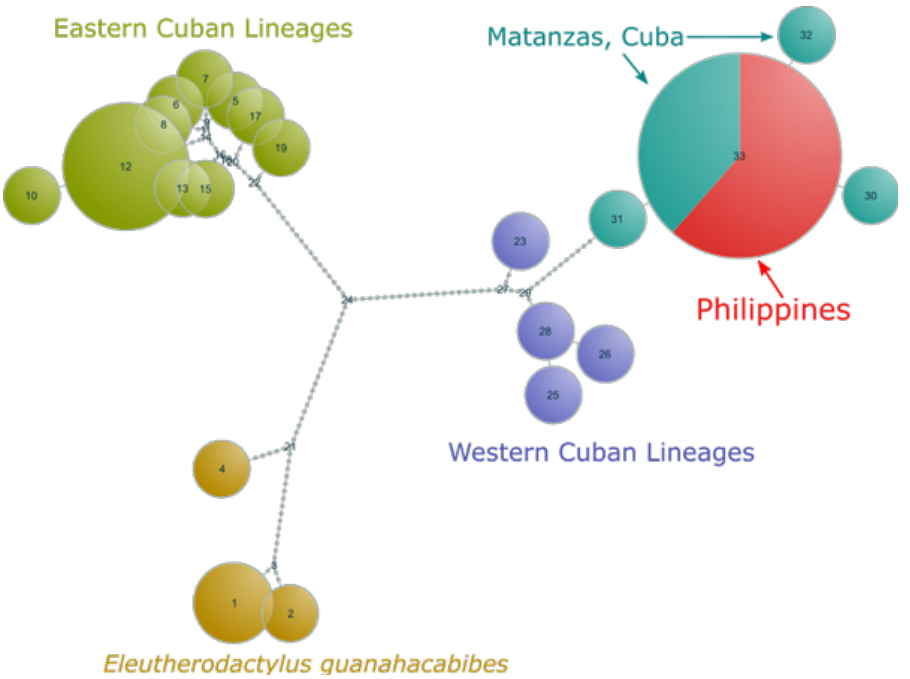

Figure 8. Haplotype network of various lineages of *E. planirostris* and *E. guanahacabibes* constructed using the python script Fitchi and following the Fitch algorithm.

that the Philippine population is more closely related to the Matanzas lineage of *E. planirostris* while Divergence-Vicariance Analysis shows that the ancestor to the Philippine population is likely from the Florida Keys or Western Cuba (yellow doughnut; Figure 9). The 16S NJ tree (Figure 5) also shows that samples from Hong Kong group with the Philippine and Florida lineages, indicating a shared origin for all locations, though the bootstrap support is rather low (60%).

S-DIVA (Figure 9) further shows that the common ancestor of the Philippine population is from Luzon. In light of the lack of samples from Mindanao and other islands where the species has been detected (Pili et al. 2019), this cannot be considered conclusive. The dispersal of the Greenhouse Frog in the archipelago will require more extensive sampling of the Philippine population.

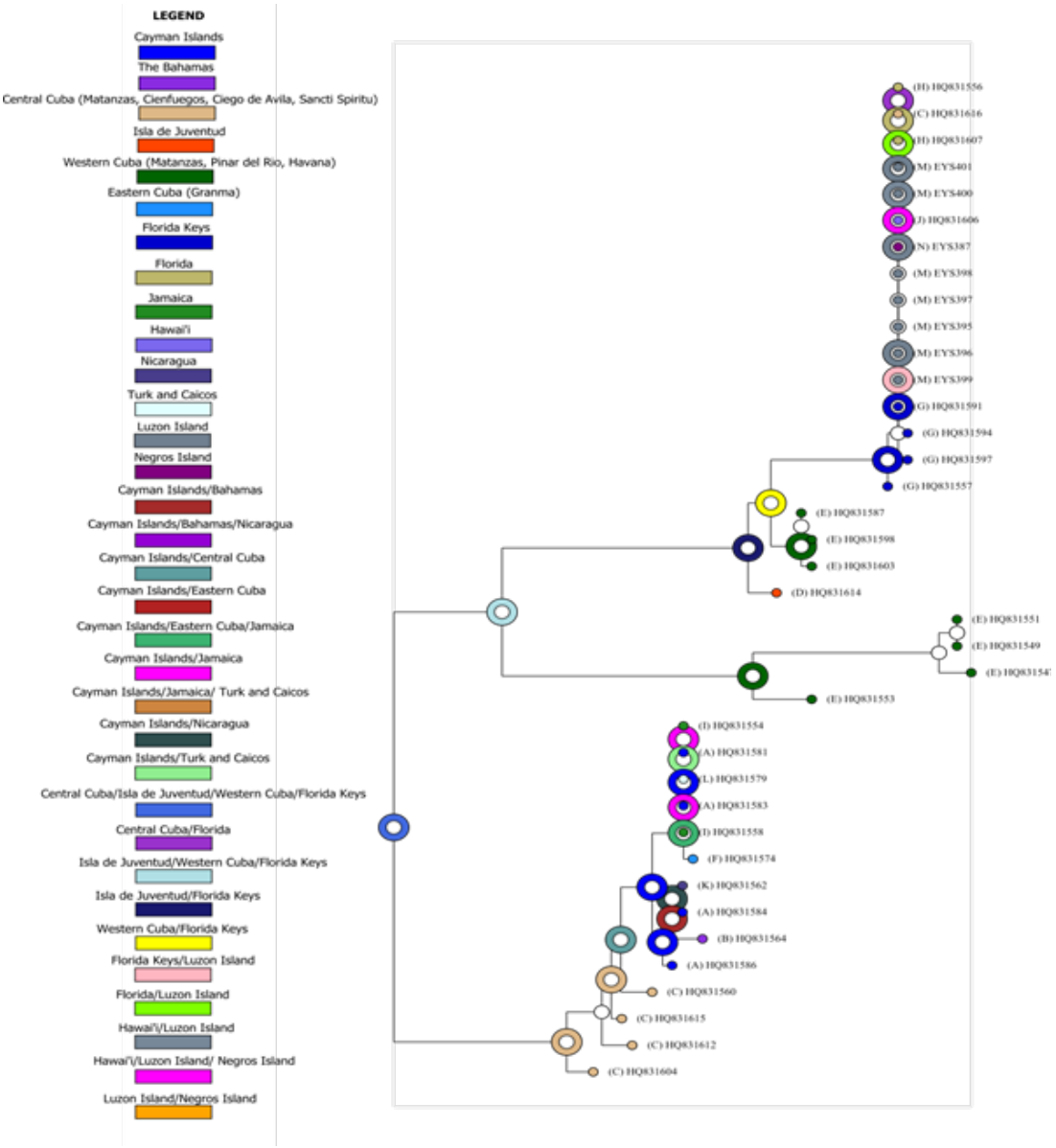

Figure 9. Statistical Dispersal Vicariance Analysis tree for *E. planirostris*. The common ancestor of the Hawai'i, Florida, and Philippine populations come either from the Florida Keys or Western Cuba.

It is suspected that the species was introduced from Florida to Hawai'i and from Hawai'i to Guam through the ornamental plant trade (Christy et al. 2007; Olson et al. 2012a). The same mode of transport is hypothesized for the Philippine population. Records from the Bureau of Plant Industry (BPI) show that ornamental plants, including bromeliads, were imported from the United States between 2005 and 2014. Since the species was first detected in Davao in October 2013 (Olson et al. 2014), it is conceivable that the species was introduced during this time period.

The Philippine population ultimately originates from Cuba and shows little variation in the sequences of three mitochondrial genes. Only one haplotype was recovered for both 16S and *cytb*. For *cox1,* there were two haplotypes, but only one individual (EYS 399) out of eight had a different haplotype. In contrast, 18 haplotypes were found for *cytb* in the species' native range (Figure 8) based on a GenBank search. Though the specimens studied here cannot be considered representative of the entire Philippine population, it suggests that the local populations might be vulnerable to the Founder Effect (Frankham 2005; Parisod et al. 2005), which might reduce the adaptability of the species to local conditions and hamper its spread in new territory (Lee 2002; Frankham 2005), especially if these populations are isolated from each other and from further waves of introductions or if future introductions are similar genetically.

Morphological measurements provide some support for the genetic evidence: the average size of Philippine *E. planirostris*, based on Snout-Vent Length (SVL), is similar to those found in Panama and Hawai'i (Table 5) (Crawford et al. 2011; Olson et al. 2012a). Furthermore, measurements of three (3) specimens in Mexico by Cedeno-Vasquez et al. (2014) gave 16, 20, and 21 mm SVL, though no distinctions between sex were made in their study. This suggests that, morphologically, Philippine specimens are similar to those found in other countries where the species is invasive (Crawford et al. 2011; Olson et al. 2012a; Cedeno-Vasquez et al. 2014). In Cuba and the Bahamas, where the species is native (Schwartz 1974; Hedges et al. 2008), Schwartz (1974) described *E. planirostris* as having a maximum SVL of 27 mm for gravid Cuban females, 20 mm for Cuban males while non-gravid females from Isla de Pinos, Cuba and New Providence, Bahamas measured 28 mm SVL; however, no mean lengths were given.

Table 5. Snout-Vent Length (SVL) in millimeters (mm) of *E. planirostris* specimens caught in other countries compared to Philippine specimens

Sex	Philippines[1]	Panama[2]	Hawai'i[3]
Male	16.47 ± 1.28	17.5	17
Female	21.31 ± 1.94	22.4	22

[1] Figures computed by authors based on five female specimens and three male specimens. No distinction was made between gravid and non-gravid females.

[2] Mean SVL for female computed by authors based upon two measurements given by Crawford et al. (2011) based on one gravid female and one non-gravid female. Figure for male is based upon one male specimen.

[3] Figures are based upon calculations made by Olson et al. (2012a) based upon a sample size of 100 for males and 176 specimens for females. No distinction was apparently made between non-gravid and gravid females.

Interestingly, Philippine specimens might still roughly follow the 3:1 ratio of striped to mottled color pattern seen in native populations of *E. planirostris* in Cuba based on field observations by one of the authors (EYS). This can only be confirmed by more extensive observations but, if so, this might suggest either a more direct origin from Florida or Cuba.

It must be noted that a low initial diversity does not always hamper invasions, as seen in the invasive populations of the American bullfrog (*Lithobates catesbeianus*) in China (Bai et al. 2012). It is therefore important to manage the spread of *E. planirostris* as early as possible. This is especially important since the species is a potential ecological threat, capable of reaching high densities (around 12,500 frogs/hectare) and consuming high numbers of insects (up to 129,000 individuals/hectare/night) based on observations in Hawai'i (Olson and Beard 2012; Olson et al. 2012a).

To reach a more definitive conclusion on the species' origin and the genetic diversity present in the introduced Philippine population, the sample base needs to be expanded. Sequences of specimens from Davao City, Mindanao, Cebu City, Cebu, and other islands where the Greenhouse Frog has been found need to be obtained in order to get a more complete picture of the genetic diversity present in the introduced Philippine population. Other parts of the country should be surveyed for additional populations of *E. planirostris*, which should also be barcoded. Barcoding could also be expanded to include nuclear genes, such as recombination-activating gene 1 (*rag1*), which has been sequenced for several *Eleutherodactylus* species, including *E. planirostris*, based on a search in GenBank.

The *cytb* (Figures 4 and 5) and 16S (Figure 6) trees, as well as the haplotype network (Figure 8), also show that *E. planirostris* is not monophyletic since *E. planirostris* clades contain *E. guanahacabibes*, a species that shares its range with Western *E. planirostris* (Hedges and Diaz 2004; Heinicke et al. 2011), though this grouping has low bootstrap support (53/76 NJ/ML, respectively, for *cytb*). The results here for *cytb* (Figures 4 and 5) are similar to the results of Heinicke et al. (2011), which also found two lineages of *E. planirostris* and possible paraphyly for the species. However, since most of the GenBank *cytb* sequences used here are from Heinicke et al. (2011), our results are not surprising. Interestingly, the analysis of Pyron and Weins (2011; Suppl. Info.) of Lissamphibia (five genes were sequenced and analyzed for *E. planirostris*: 12S, 16S*, h3a, rhod, sia,* and *tyr*) showed that *E. guanahacabibes* diverges from a clade formed by *E. planirostris* and *E. casparii*, contrary to the findings of this study and of Heinicke et al. (2011) using *cytb* sequences. This suggests that sampling more genes might be needed to fully resolve the phylogeny of the *E. planirostris* species group.

For 16S (Figure 6), the phylogeny is more problematic. *Eleutherodactylus guanahacabibes* is shown to cluster with sequences that represent Eastern *E. planirostris* (Crawford et al. 2011), albeit with low bootstrap support (42 % NJ). Also, the sequence from Havana, Cuba (JF769005) that is supposed to represent Western *E. planirostris* (Crawford et al. 2011) does not group with either Western or Eastern *E. planirostris*, and this divergence has moderate bootstrap support (60% NJ). A greater sample size for 16S, with sequences coming from various geographic regions, might help clarify the lineages present in *E. planirostris* with respect to the 16S gene and verify the groupings found using *cytb*.

Eight *cox1* sequences for *E. planirostris* will be added to GenBank as part of this study. While of interest by themselves since they represent a new geographic location for *E. planirostris*, they also add to the database of *cox1* barcodes of amphibian taxa currently present in GenBank and other databases, such as the Barcode of Life Database (BOLD). They can be used for further studies of amphibians, including studies similar to that presented here.

In summary, the eight specimens of *E. planirostris* barcoded in this study showed minimal genetic diversity amongst themselves based on sequences of three mitochondrial genes (*cytb*, 16S, and *cox1*). Analysis of the *cytb* phylogenetic tree showed that the Philippine samples grouped with the GenBank sequences of *E. planirostris* specimens from Matanzas province of Cuba, Hawai'i, and Florida. For 16S and *cox1*, the Philippine population grouped with specimens from Florida

and Panama. The ultimate origin of the introduced Philippine population is Matanzas, Cuba, though the immediate origin is more likely to be Hawai'i, Florida or neighboring Asian regions with introduced *E. planirostris* populations such as Hong Kong, considering the mode by which this species is thought to have spread. The low genetic diversity of local populations of *E. planirostris* suggests that it may be vulnerable to the Founder Effect, especially if these populations become isolated.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTION OF INDIVIDUAL AUTHORS

EYS obtained specimens and scored morphometric data. EYS, IKCF, and GCLQ conceptualized the study and experimental procedure. GCLQ performed the laboratory work and computer analysis. All authors analyzed the results and wrote the manuscript.

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