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Genetic diversity of *Oophaga vicentei* (Anura: Dendrobatidae) and taxonomic position of a remarkable color morph from Panama

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The genus *Oophaga* Bauer, 1994 comprises 12 species and is harbored among Neotropical poison frogs of the Dendrobatidae family (Frost 2023). These species are distributed throughout Central America and northwestern South America (Grant et al. 2017). Despite being one of the most studied Neotropical anuran groups, the phylogenetic relationships, taxonomic status and species boundaries of this genus remain unclear. The known color polymorphism within and across species poses challenges for accurate taxa identification, emphasizing the importance of integrative taxonomic tools (Brown et al. 2011).

Oophaga vicentei (Jungfer, Weygoldt & Juraske, 1996) is distributed in the forested areas of the Coclé and Veraguas provinces in the central region and the Caribbean of Panama. It inhabits altitudes ranging from sea level up to approximately 900 meters above sea level (m a.s.l.) and is exclusively found on epiphyte-laden trees within preserved forests (Lötters et al. 2007). Several color morphs have been described for this species, including green, blue, and bright red, with patterns of dorsal dark markings including bands, spots, and vermiculations (Lötters et al. 2007). Until the study conducted by Flores et al. (2019), the field ecology of this species was basically unknown. Their research provided valuable insights into the calling activity, bioacoustics and diet of males from three different populations in Central Panama.

During fieldwork surveys on June 2022 in Panama, aimed at identifying divergent color morphs of *Oophaga vicentei*, three of us (RI, VMO, AR) visited two localities in the Colón and Veraguas provinces. In one of them, we observed individuals displaying a unique color pattern characterized by pale yellow dots distributed on a dark brown background in the dorsal region (Fig. 1). This color pattern bears a resemblance to *Oophaga arborea* (Myers, Daly & Martínez, 1984), another endemic species from Panama. Around 2004, specimens of this dotted morph were smuggled into the European pet trade market and sold as *O. arborea* but later classified as *O.* cf. *vicentei* (Ostrowski & Mahn 2023). In order to elucidate the taxonomic position of this population, as well as the other collected samples, herein we use DNA sequence information to determine their phylogenetic relationships and provide insights into the genetic diversity and ecology of this population.

We sampled frogs at the locality of La Empalizada, Provincia de Veraguas, Panamá (8°45'40" N, 81°12'50" W, 82 m a.s.l.) and at the surroundings of La Ceiba, Área de Uso Múltiple Donoso, Distrito Especial Omar Torrijos Herrera, Provincia de Colón, Panamá (8°48'30" N, 80°36'24" W, 100 m a.s.l.) (Fig. 1A). We searched for frogs in the leaf litter, trunks, vines and leaves aided by their vocalizations and captured individuals by hand. We photographed the collected individuals in the field and later euthanized them by immersion in 5% MS-222. We subsequently extracted thigh muscle tissue and stored it in 96% ethanol vials.

Once in the lab, we extracted total DNA from the tissues using Qiagen DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) and PCR amplified two commonly used mitochondrial markers in amphibians, the 16S rRNA (16S)

and the protein-coding cytochrome b gene (COB). We performed the PCRs with a final volume of 11 µl using: 7 µl sterile H_2O, 2.5 µl GoTaq Reaction buffer (Promega), 0.3 µl each primer, 0.25 µl dNTP (10 mM), and 0.08 µl GoTaq® DNA polymerase (Promega). For 16S amplification, we used the primers 16Sar-L and 16Sbr-H (Palumbi et al. 1991; respectively 5'-CGCCTGTTTATCAAAAACAT-3' and 5'-CCGGTCTGAACTCAGATCACGT-3'). The PCR program included an initial denaturing step of 3 min at 95°C followed by 35 cycles of amplification (95°C for 20 s; 50°C for 20 s of annealing temperature; and 65°C for 1 min for extension), with a final extension step at 65°C for 3 min. Additionally, for COB amplification, we used the primers cytbA and cytbC (Bossuyt & Milinkovitch 2000; respectively 5'-CCATGAGGACAAATATCATTYTGRGG-3' and 5'-CTACTGGTTGTCCTCCGATTCATGT-3'). The PCR cycling was identical except for the 38 cycles of amplification (95°C for 20 s; 52°C for 20 s of annealing temperature; and 68°C for 50 s for extension) and the final extension step at 72°C for 3 min. Sanger sequencing in both directions was performed by Eurofins Genomics Europe Sequencing GmbH (Köln, Germany). We inspected and edited the chromatograms for quality and assembled the sequences using Geneious software (v.10.3, Biommaters). The newly obtained sequences were deposited in GenBank (NCBI accession numbers: OR133808-15 and OR271415-22).

For each of the two loci, we constructed multiple sequence alignments including the newly obtained sequences plus homologous sequences available on NCBI GenBank. Our compilation included DNA sequences from eight *Oophaga* species from Central (*O. arborea*, *O. granulifera*, *O. pumilio*, *O. speciosa*, *O. vicentei*) and South America (*O. histrionica*, *O. lehmanni*, *O. sylvatica*). Due to the unavailability of homologous 16S and COB sequences, *O. anchicayensis*, *O. andresi*, *O. occultator*, and *O. solanensis* could not be included in the alignments. In accordance with recent phylogenetic reconstructions (Guillory et al.

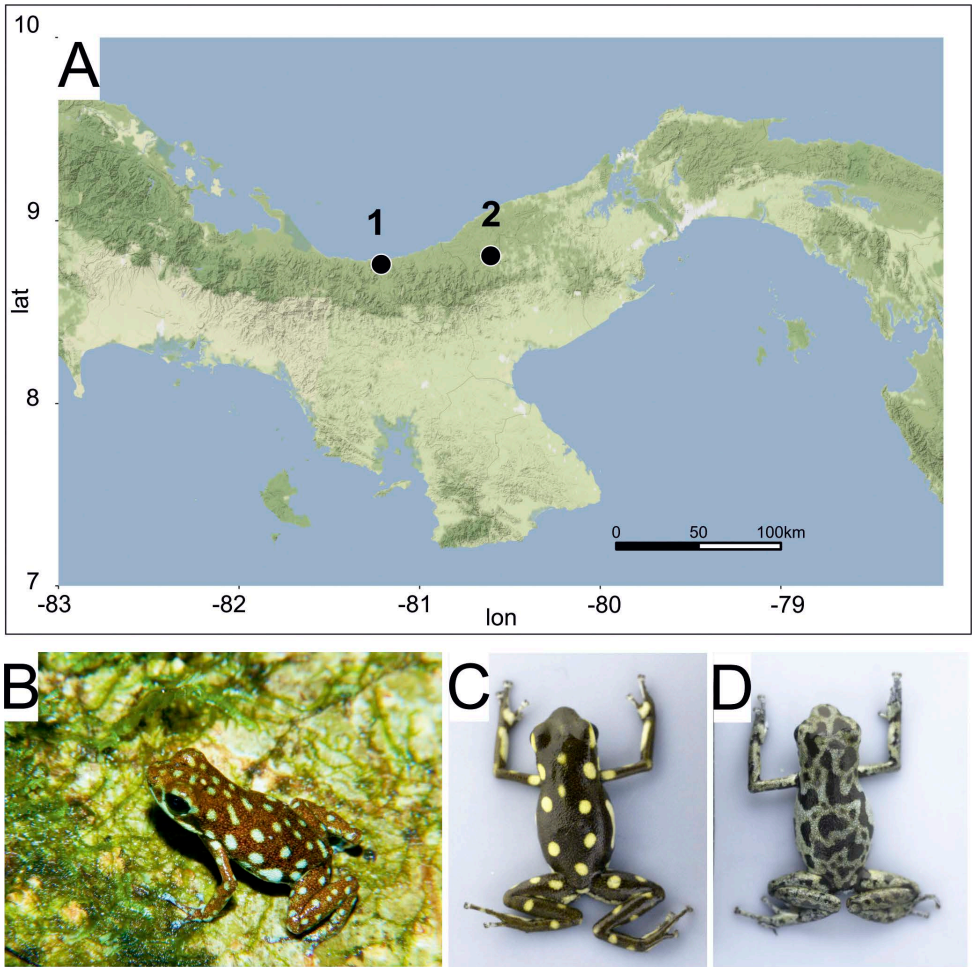

Figure 1. Geographic location and representative photographs of the study populations of *Oophaga vicentei*. (A) Map of Western Panama identifying the study sites: (1) La Empalizada, Veraguas; (2) La Ceiba, Área de Uso Múltiple Donoso, Colón. (B) Male of *O. vicentei* from La Empalizada photographed in situ by AR; (C) dorsal view of *O. vicentei* male from La Empalizada, photographed by VMO; and (D) dorsal view of *O. vicentei* male from La Ceiba, photographed by VMO.

2019, Jetz & Pyron 2018), sequences from three *Dendrobates* species (*D. auratus*, *D. leucomelas*, *D. tinctorius*) were included as outgroups (GenBank accession numbers are provided in Appendix 1). Alignments of the 16S and COB sequence sets were conducted in Mega using default settings (v.5.0, Tamura et al. 2011) and later concatenated using the R package concatipede (v.1.0.0, Vecci & Bruneaux 2021). We inferred the phylogenetic relationships of the samples in the concatenated alignment using iQTree (v.1.6.12, Nguyen et al. 2015). We implemented four data partitions (16S, and each of the three COB codon positions) and searched for the best-fit models of sequence evolution and partition scheme with the Model Finder algorithm of iQTree. The model fit results (16S and all COB codons together: TPM2+G4) were used in the final maximum likelihood (ML) tree reconstruction. Node support was assessed by an ultrafast bootstrap with 1000 pseudoreplicates.

We observed approximately a dozen active individuals at La Empalizada site, only five of these were collected. Most individuals we were able to locate were active calling males, perching on tree trunks, vines, rocks, and the leaf litter, from the ground to 3 m high. All observed individuals exhibited a distinctive dorsal color pattern with pale yellow to light green spots over a dark background (Fig. 1B, C). The ventral color was pale yellow to light green with no distinctive markings or dark areas in the gular region. At La Ceiba, the understory vegetation had been partially removed. However, active frogs including calling males were similarly seen (from the ground to 3 m high), on the leaf litter and on superficial roots, trunks and low branches of trees. Further, some males were heard calling from perches above 3 m. In total, eight males were collected at this locality. The individuals displayed dark brown and blueish green color with vermiculation pattern on the dorsal area (Fig. 1D). The ventral area was light blueish green without patterns. As our sampling protocol included dissection and extraction of multiple organs, no voucher specimens were obtained.

The concatenated alignment of 16S and COB DNA sequences measured 1,515 bp in length and included 42 terminals (eight obtained in this study; Appendix 1). The reconstructed ML phylogeny displayed a strongly supported (100% ultrafast bootstrap value) *O. vicentei* as monophyletic clade encompassing all newly obtained sequences from La Empalizada and La Ceiba plus the previously existing GenBank record of *O. vicentei* from El Copé (Grant et

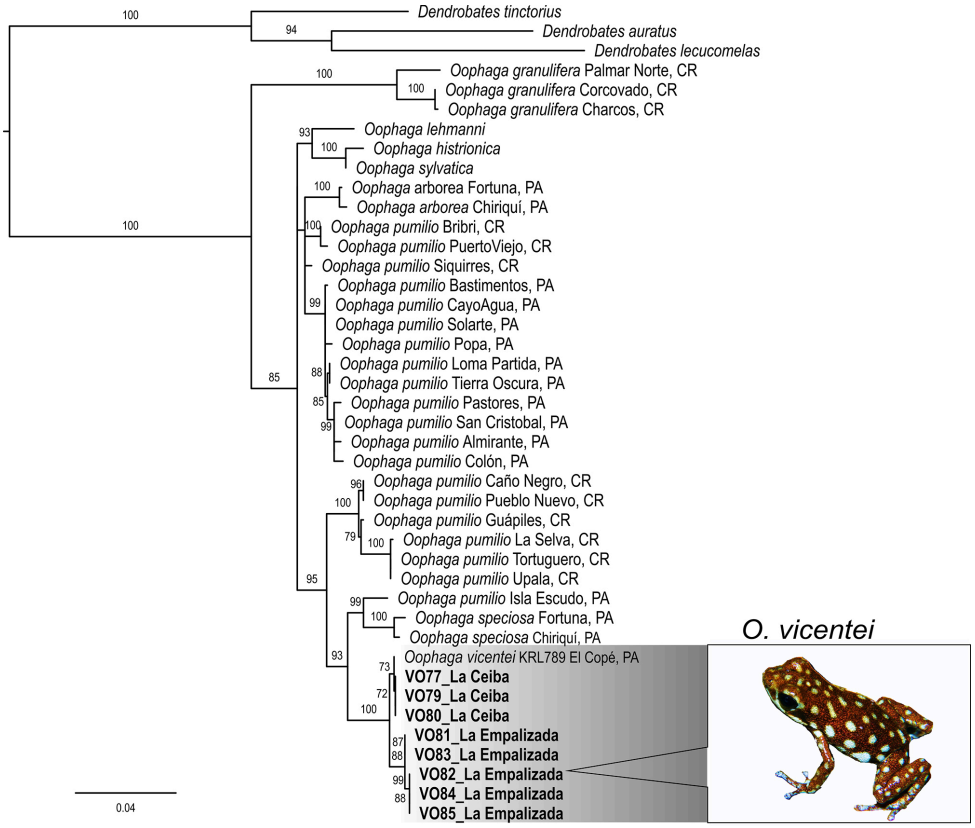

Figure 2. Maximum likelihood phylogenetic tree obtained from a matrix of 1,515 bp of mitochondrial DNA sequences of 42 terminals of *Oophaga* and *Dendrobates* (outgroup) frogs. Ultrafast bootstrap support values above 70% are presented on the tree branches. The *O. vicentei* clade is highlighted in gray, with the newly obtained sequences in bold. The inset shows a photograph of a male with the dotted pattern observed at La Empalizada locality. Abbreviations: CR = Costa Rica, PA = Panama. Sequence accession numbers are provided in Appendix 1.

al. 2006). This clade clustered within a strongly supported clade including northern Costa Rica and Isla Escudo de Veraguas samples of *O. pumilio* and *O. speciosa*. Interestingly, the geographically proximal GenBank accessions of *O. pumilio* from Bocas del Toro region were not in this clade and their phylogenetic position, as well as those of *O. pumilio* terminals from Southern Costa Rica, *O. arborea*, and the South American *Oophaga* were not resolved in the ML tree (Fig. 2).

The genus *Oophaga* has been one of the most studied amphibian groups in terms of behavior, ecology, evolutionary biology and aposematism (e.g. Wang & Shaffer 2008, Brown et al. 2010, Yang et al. 2019). Despite this, the phylogenetic relationships, taxonomic status, and species boundaries remain unclear, resulting in the grouping into species complexes. By including sequences from multiple samples, our phylogenetic analysis provides the first molecular evidence for the monophyly of *O. vicentei* and its close relationship with *O. speciosa* and the *O.* cf. *pumilio* from Isla Escudo de Veraguas. Additionally, our mitochondrial phylogeny corroborates the notion that *O. pumilio* is a paraphyletic taxon comprising distinct mitochondrial lineages some of which show uncertainties in their phylogenetic relationships. Similar inconsistencies in mitochondrial phylogenies have been previously reported by multiple authors (Hagemann & Pröhl 2007, Hauswaldt et al. 2011, Grant et al. 2017), and reinforce the need for taxonomic reassessment of this species complex. It is also worth noting that our taxa and loci sampling was geared towards the elucidation of the relationship of *O. vicentei* samples. In order to reconstruct the phylogeny of this genus a more extensive sampling, of loci and taxa, will be required.

The pronounced variations in coloration observed among the localities with available DNA sequences and herein studied (La Empalizada: dark with light dots, La Ceiba: bluish green with dark vermiculations, and El Copé: yellowish green with variable spotting) (Jungfer et al. 1996), in complement with the existence of other less known localities where red, green and blue color morphs have been documented (AmphibiaWeb 2023, Ostrowski & Mahn 2023) underscore the potential of this species for future investigations into the evolution of color polymorphism. A comprehensive characterization of the phenotypic and genotypic diversity of *O. vicentei* in mainland Panama will also allow for additional testing of the existing hypothesis for the evolution of the polytypic *O. pumilio* in the Bocas del Toro archipelago.

Apart from elucidating the phylogenetic position of a unique color morph of *O. vicentei*, our field-based behavioral observations contribute further insights into the ecology of this species. Typically, *O. vicentei* is considered an exclusive tree-dweller associated with bromeliads in the arboreal stratum (Lötters et al. 2007), spending most of the time high in the upper canopy (Flores et al. 2019). However, at the La Empalizada and La Ceiba localities, we observed active males of this species calling from the leaf litter and perches in the lower stratum of the forest understory. These observations, together with the Peña et al. (2016) report, who found a female in the forest floor in another locality within the Santa Fe area, suggest that microhabitat preferences in *O. vicentei* may exhibit plasticity and be locality-specific across the range of occurrence.

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Appendix 1

GenBank accession numbers of DNA sequences of *Oophaga* and *Dendrobates* used in this study with newly obtained sequences highlighted in bold. Abbreviations: CO = Colombia, CR = Costa Rica, EC = Ecuador, and PA = Panama.

Species	sample	16S	COB
O. vicentei	VO77_La Ceiba	**OR133808**	**OR271415**
O. vicentei	VO79_La Ceiba	**OR133809**	**OR271416**
O. vicentei	VO80_La Ceiba	**OR133810**	**OR271417**
O. vicentei	VO81_La Empalizada	**OR133811**	**OR271418**
O. vicentei	VO82_La Empalizada	**OR133812**	**OR271419**
O. vicentei	VO83_La Empalizada	**OR133813**	**OR271420**
O. vicentei	VO84_La Empalizada	**OR133814**	**OR271421**
O. vicentei	VO85_La Empalizada	**OR133815**	**OR271422**
O. vicentei	KRL789_El Cope, PA	DQ502167	DQ502602
O. speciosa	Fortuna, PA	AF098747	AF120014
O. speciosa	Chiriquí, PA	DQ502037	DQ502468
O. arborea	Fortuna, PA	AF098748	AF120015
O. arborea	Chiriquí, PA	DQ502036	DQ502467
O. pumilio	Bribri, CR	EF597161	EF597201
O. pumilio	Cano Negro, CR	EF597162	EF597202
O. pumilio	Guapiles, CR	EF597163	EF597203
O. pumilio	La Selva, CR	EF597164	EF597204
O. pumilio	Pueblo Nuevo, CR	EF597165	EF597205
O. pumilio	Puerto Viejo, CR	EF597166	EF597206
O. pumilio	Siquirres, CR	EF597167	EF597207
O. pumilio	Tortuguero, CR	EF597168	EF597208
O. pumilio	Upala, CR	EF597169	EF597209
O. pumilio	Almirante, PA	EF597170	EF597210
O. pumilio	Bastimentos, PA	EF597171	EF597211
O. pumilio	Colón, PA	EF597172	EF597212
O. pumilio	Cayo Agua, PA	EF597173	EF597213
O. pumilio	Escudo, PA	EF597174	EF597214
O. pumilio	Loma Partida, PA	EF597175	EF597215
O. pumilio	Pastores, PA	EF597176	EF597216
O. pumilio	Popa, PA	EF597177	EF597217
O. pumilio	San Cristobal, PA	EF597178	EF597218
O. pumilio	Solarte, PA	EF597179	EF597219
O. pumilio	Tierra Oscura, PA	EF597180	EF597220
O. granulifera	Corcovado, CR	AF098749	AF120016
O. granulifera	Charcos, CR	HE804764	HE775251
O. granulifera	Palmar Norte, CR	HE804766	HE775254
O. histrionica	unknown	AF098742	AF120009
O. lehmanni	Valle del Cauca, CO	DQ502034	DQ502465
O. sylvatica	Santo Domingo, EC	DQ502059	DQ502490
D. auratus	unknown	MF069434	MF069434
D. leucomelas	unknown	MF069435	MF069435
D. tinctorius	unknown	MF069437	MF069437