



Taxonomy of Peruvian gladiator frogs of the *Boana pulchella* species group (Anura: Hylidae) revisited: new insights from DNA sequences, bioacoustics and morphology

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Manuscript received: 15 July 2026

Accepted: 25 August 2026 by STEFAN LÖTTERS

Abstract. Based on newly collected specimens, tissue samples for molecular genetic analyses, and recent field recordings of advertisement calls, we reevaluate the taxonomic status of Peruvian species of the *Boana balzani* clade in the *B. pulchella* species group. Among the five nominal species recognized in this clade, our analyses corroborate the species status of *B. balzani*, *B. gladiator*, and *B. melanopleura*. In contrast, given their monophyly, low genetic divergence in the mitochondrial 16S rRNA gene, absence of species-specific differences in advertisement calls, and lack of diagnostic morphological traits, we conclude that *B. aguilari* and *B. palaestes* are conspecific. Consequently, we consider *Hypsiboas aguilari* LEHR, FAIVOVICH & JUNGFER, 2010 a junior synonym of *Boana palaestes* (DUELLMAN, DE LA RIVA & WILD, 1997), and update the current knowledge about the species' geographical ranges.

Key words. Amphibia, *Boana balzani* clade, genetic divergence, advertisement calls, morphological variation, synonymy, Peru.

Introduction

Andean gladiator frogs of the *Boana pulchella* species group, family Hylidae, received considerable attention from researchers during the last decades (e.g., DUELLMAN et al. 1997, FAIVOVICH et al. 2004, 2005, 2021, KOSCINSKI et al. 2008, KÖHLER et al. 2010, LEHR et al. 2010, 2011, PINHEIRO

et al. 2016, 2024, VASCONCELLOS et al. 2021, MARINHO et al. 2022, NAKAMURA et al. 2025). According to the most recent multi-locus molecular phylogenetic analysis and systematic revision by FAIVOVICH et al. (2021), the Andean members of this species group are recovered in two well-supported clades: (1) the southern Andean *Boana riojana* clade, including *B. callipleura* (BOULENGER, 1902), *B. marianitae*

(CARRIZO, 1992), and *B. riojana* (KOSLOWSKY, 1895); and (2) the central Andean *Boana balzani* clade, including *B. aguilari* (LEHR, FAIVOVICH & JUNGFER, 2010), *B. balzani* (BOULENGER, 1898), *B. gladiator* (KÖHLER, KOSCINSKI, PADIAL, CHAPARRO, HANDFORD, LOUGHEED & DE LA RIVA, 2010), *B. melanopleura* (BOULENGER, 1912), and *B. palaestes* (DUELLMAN, DE LA RIVA & WILD, 1997).

Within the *Boana balzani* clade, FAIVOVICH et al. (2021) found comparatively low uncorrected p-distances in DNA sequences of the mitochondrial 16S rRNA gene between *B. aguilari*, *B. palaestes*, and *B. gladiator*, and pointed to several uncertainties involved in the diagnoses of *B. aguilari* and *B. gladiator* with respect to *B. palaestes*. These uncertainties relate to morphological characters as well as to differentiation in advertisement calls. FAIVOVICH et al. (2021) demonstrated that the call described for *B. palaestes* by DUELLMAN et al. (1997) corresponds to a centrolenid species, thus explaining the remarkable call differences used as a distinguishing character in the respective diagnoses of *B. aguilari* and *B. gladiator* (LEHR et al. 2010, KÖHLER et al. 2010). The actual call of *B. palaestes* is structurally similar to calls known from the *B. balzani* clade, namely consisting of a long, pulsed note (FAIVOVICH et al. 2021). However, proper call comparisons were hampered by the fact that calls of *B. aguilari* (as well as those of *B. melanopleura*) were recorded in captivity (LEHR et al. 2010). The calls of *B. aguilari* had overlapping values in temporal and spectral parameters with those of *B. palaestes*, but their spectrograms appeared somehow different when compared visually. Therefore, FAIVOVICH et al. (2021) highlighted the need to obtain proper field recordings to reevaluate the advertisement call characters of *B. aguilari*, with supposed call differences probably being the only evidence for recognizing *B. aguilari* as distinct from *B. palaestes*. In addition, the only morphological difference between adults of *B. aguilari* and *B. palaestes* mentioned by LEHR et al. (2010) is the presence of white spots on flanks in *B. aguilari*, absent in *B. palaestes* according to its original description provided by DUELLMAN et al. (1997).

Recent fieldwork along the eastern Andean versant in central and southern Peru resulted in the accumulation of additional data, namely specimens, tissue samples for molecular genetic analyses, and call recordings. With these new data at hand, we reevaluate the taxonomic status of certain nominal species in the *B. balzani* clade, properly characterize their advertisement calls, and update their known distribution.

Material and methods

Fieldwork and voucher specimens

Fieldwork was conducted in different areas along the eastern Andean slopes in central and southeastern Peru. Specimens were observed and collected during opportunistic searches at night using torchlights and headlamps. Collected individuals were anesthetized by applying 5% lidocaine or 20% benzocaine gel on their ventral surfaces, and

ethanized with an overdose of the same substances (McDIARMID 1994). Tissue samples were taken prior to fixation and stored in pure ethanol, while specimens were fixed using 96% ethanol or formalin and subsequently stored in 70% ethanol. Newly collected specimens were deposited in the herpetological collections of the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos (MUSM), Lima, Peru; Museo de Biodiversidad del Peru (MUBI), Cusco, Peru; Pro Fauna Silvestre, Ayacucho, Peru (PFAUNA), and Centro de Ornitología y Biodiversidad, Lima, Peru (CORBIDI). KU refers to University of Kansas, Museum of Natural History, Division of Herpetology, Lawrence, Kansas, USA. Other museum abbreviations follow those listed by FROST (2026). AC refers to field numbers of A. CATENAZZI. Geographic position was recorded using handheld GPS receivers set to WGS84 datum or with modern smartphones and later verified with Google Earth.

Molecular genetics

Character sampling: For every newly available sample identified as *Boana aguilari*, *B. gladiator*, *B. melanopleura*, or *B. palaestes* we sequenced a fragment of the mitochondrial 16S rRNA gene delimited by the primers 16SarL (PALUMBI et al. 1991) and 16S-WilK2 (WILKINSON et al. 1996); for a few of them we also completed the 12S-tRNA^{Val}-16S rRNA fragment with the same primers employed by FAIVOVICH et al. (2005). These sequences were added to the dataset for the *B. pulchella* group produced by FAIVOVICH et al. (2021) as modified by NAKAMURA et al. (2025), who added *B. cymbalum* and the available 28S sequences for species of the group and outgroups. This dataset already contained 23 specimens of the five nominal species included in the *B. balzani* clade, and includes portions of the mitochondrial genes encoding cytochrome b (Cytb), COI, 12S, tRNA^{Val}, 16S, and a fragment including the complete upstream section of 16S, tRNA^{Leu}, NADH dehydrogenase subunit 1 (ND1), and tRNA^{Ile}, and the nuclear genes are portions of seven in absentia homolog 1 (SIAH1), exon 1 of rhodopsin (RHO), exon 1 of tyrosinase (TYR), single exon of Recombination Activating 1 (RAG-1), exon 2 of CXCR4 and 28S rRNA. See FAIVOVICH et al. (2021: suppl. data 1) and NAKAMURA et al. (2025: suppl. material 2) for GenBank accession numbers of sequences of the dataset of these authors employed in the study herein.

DNA isolation and sequencing: Procedures for the majority of new samples used are those described by CASTILLO-URBINA et al. (2025) and CHÁVEZ et al. (2025), and for the other part of new samples the following steps were used: Whole cellular DNA was extracted from ethanol-preserved tissues with the DNeasy (QIAGEN, Valencia, CA) isolation kit or standards high-salt protocol. Amplification was performed in a 25-μl-volume reaction using TAQ DNA polymerase and reagents (Thermo Fisher Scientific, Massachusetts, USA) or Taq DNA Polymerase master mix (Ampliqon A/S, Denmark). For all the amplifications, the PCR program included an initial denaturing

step of 30 seconds at 94 °C, followed by 35 cycles of amplification (94 °C for 30 seconds; 48 °C for 30 seconds; 60 °C for 60 seconds), with a final extension step at 72 °C for 6 minutes. Polymerase chain reaction (PCR) amplification products were cleaned using Exonuclease I and Alkaline phosphatase (Thermo Fisher Scientific, Massachusetts, USA) and sequenced using fluorescent dye-labels terminators (ABI Prism Big Dye Terminators v. 1.1 cycle sequencing kits; Applied Biosystems, Foster City, CA) with an ABI 3730XL (Applied Biosystems, Foster City, CA); all samples were sequenced in both directions to check for potential errors. Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software Sequencher 5.0 (Gene Codes, Ann Arbor, MI). All new sequences were submitted to GenBank (accession numbers PZ815409–PZ815451); the respective specimen metadata along with the accession numbers are provided in the public Zenodo repository under <https://doi.org/10.5281/zenodo.21371652>.

Sequences were aligned using the online software MAFFT v7 (KATO & TOH 2008, KATO et al. 2019) under the strategy E-INS-i with default parameters for gap opening and extension. These alignments were used for both phylogenetic analyses and clade support estimations. Alignments were visualized with BioEdit (HALL 1999).

Phylogenetic analysis: For the maximum parsimony phylogenetic analyses, we employed TNT v1.6 (GOLOBOFF & MORALES 2023). Searches used the command “New Technology Search” under search level 50, which combines the Parsimony Ratchet, Tree Drifting, Sectorial Searches, and Tree Fusing (GOLOBOFF 1999, NIXON 1999). For this driven search, we used the default options for these algorithms, hitting the most parsimonious trees 300 times. The resulting trees were submitted to a round of TBR branch swapping. Searches were done under the collapsing option “minimum length”, which collapses every node whose minimum length is 0. Parsimony jackknife absolute frequencies (FARRIS et al. 1996) were calculated in TNT v1.6 using new technology requesting 3 hits with driven searches under search level 15, for a total of 1000 replicates. Gaps were treated as a fifth state, but to enable comparisons with the maximum likelihood analysis (see below), we also performed the analyses with gaps treated as missing data. For the graphic tree edition, we used the TNT script *forfai.run* written by P. A. GOLOBOFF (available at <https://www.lillo.org.ar/phylogeny/tnt/scripts/forfai.run>), which takes as input the most parsimonious trees and the trees resulting from the resample pseudoreplicates, and outputs a strict consensus with branch lengths and support values in Newick format. This file was read with FigTree v1.4.3 (RAMBAUT 2014) for visualization and editing.

The maximum likelihood phylogenetic analysis was performed with IQ-TREE v2.2.0 (MINH et al. 2020) using the same dataset as under parsimony and gaps treated as missing data. The best partition scheme and models were selected using ModelFinder (KALYANAMOORTHY et al. 2017) as implemented in IQ-TREE (NGUYEN et al. 2015). To thoroughly explore tree space, we conducted 10 independ-

ent tree searches with perturbation strengths ranging from 0.1 to 0.5 (option *-pers* 0.1–0.5), where these values control the degree of topological rearrangements during the search process. The maximum likelihood searches were iterated until the best log-likelihood value remained unchanged for 1000 consecutive cycles (option *-nstop* 1000). The maximum likelihood analysis was conducted with 1000 ultrafast bootstrap replicates (MINH et al. 2013, HOANG et al. 2018) using the option *-bnni* which reduces the risk of overestimating branch supports caused by severe model violations. The resulting tree (from the best scoring run with the highest log-likelihood value) was visualized and edited in FigTree v1.4.3 (RAMBAUT 2014).

Sequence divergence: Uncorrected pairwise sequence distances (UPD) were calculated in MEGA 11 (STECHEER et al. 2020, TAMURA et al. 2021) for the 16S rRNA gene fragment delimited by the primers 16Sar-L and 16S-Wilk2, using the option pairwise deletion for gaps and missing data.

Bioacoustics

Vocalizations were recorded in the field using different analog and digital devices. These include Uher, Sony WM-D6C, Olympus LS-5, Tascam DR05, and Zoom H6. In some cases, directional microphones were attached to the devices (e.g., Uher, Sennheiser Me-80, Zoom SSH6), whereas in other cases built-in microphones were used. Recordings obtained with digital devices were recorded at 44.1 kHz and 16-bit resolution, saved as uncompressed wave format on SD cards. Recordings were sampled or re-sampled at 22.05 kHz and 32-bit resolution and analyzed using the software Cool Edit Pro 2.0. Temporal parameters were measured in the oscillograms. Frequency information was obtained through Fast Fourier Transformation (FFT; width 1024 points) at Hanning window function. Spectrograms were produced at Blackman window function with 256 bands resolution. For a clearer graphical presentation of audiospectrograms, in most cases, careful band-pass filtering was performed to remove background sounds, applied only to frequencies outside the prevalent bandwidths of calls. Temporal measurements are summarized as range with mean $\pm$ standard deviation in parentheses. Description, terminology, and methods follow those recommended by KÖHLER et al. (2017), using the note-centered terminological scheme. All recordings treated herein were newly analyzed using the exact same methodology, even if call descriptions derived from the same recordings were published previously. We did not consider call repetition rate as a parameter, as it is highly dependent on calling motivation (see KÖHLER et al. 2017) and because in several recordings multiple males are calling in a chorus, making it nearly impossible to allocate certain calls to a single individual. The recording obtained of *Boana palaestes* at its type locality (near Tutumbaro, Ayacucho, Peru) in 1975 by W. E. DUELLMAN contains calls of more than one individual. Similarly to the description provided by FAIVOVICH et al. (2021), we here analyzed only those calls which sounded

regularly motivated and had the highest relative amplitude in that respective recording. Representative cuts of call recordings were archived in the Zenodo repository under <https://doi.org/10.5281/zenodo.21371652>.

Morphology

We focused on examination of morphological characters in specimens referred to as *Boana aguilari* ($n = 20$) and *B. palaestes* ($n = 33$). Among these were specimens of the respective type series deposited in the KU and MUSM collections, plus newly collected material, including topotypic specimens (see Appendix). Coloration was qualitatively evaluated in preserved specimens and partly in living individuals based on digital photographs. Morphological measurements were taken with digital calipers rounded to the nearest 0.1 mm (not presented here, see below).

Results

Phylogeny and sequence divergence

The newly added specimens of *Boana aguilari*, *B. gladiator*, *B. melanopleura*, and *B. palaestes* were mostly recovered with those of the respective species included in the dataset of FAIVOVICH et al. (2021) as modified by NAKAMURA et al. (2025). As in these studies, all the analyses performed here recover a well-supported *B. balzani* clade (97% jackknife, 100% ultra-fast bootstrap), with *B. balzani* + *B. melanopleura* (98% jackknife, 92% ultra-fast bootstrap) being the sister taxon of a clade (99% jackknife, 100% ultra-fast bootstrap) that includes *B. gladiator*, as the sister taxon of a clade (86% jackknife, 98% ultra-fast bootstrap) that includes *B. aguilari* and *B. palaestes* (Fig. 1; see <https://doi.org/10.5281/zenodo.21371652> for complete topologies of parsimony and maximum likelihood analyses). In the *B. aguilari* + *B. palaestes* clade, we recover a specimen of *B. palaestes* from Puente San Miguel (2156 m a.s.l.), La Convención, Cusco department (CORBIDI 29695), as the sister of a clade (< 50% jackknife; 81% ultra-fast bootstrap) including all specimens of *B. aguilari* (76% jackknife; 100% ultra-fast bootstrap) and all other specimens of *B. palaestes* (50% jackknife; 81% ultra-fast bootstrap). Another available *B. palaestes* sample from a nearby locality, Alto Shimaá (1701 m a.s.l.), La Convención, Cusco department (MUBI 17571), is recovered in a polytomy (parsimony) with, or as the sister (maximum likelihood, 81% ultra-fast bootstrap) of the other specimens of *B. palaestes*.

Uncorrected p-distances (UPD) in the 16S rRNA gene between the three nominal species *B. aguilari*, *B. gladiator* and *B. palaestes* are 1.19–3.67%. UPD between *B. aguilari* and *B. palaestes* are 1.19–2.36%, and all samples of *B. gladiator* have UPD of 2.11–3.67% to the clade including *B. aguilari* and *B. palaestes* samples (with exception of sample CORBIDI 29695; see below). *Boana balzani* has UPD of 3.51–5.42% to *B. aguilari*/*B. palaestes*/*B. gladiator*, and 3.62–4.86% to *B. melanopleura*. The latter species

has 4.70–7.11% UPD to *B. aguilari*/*B. palaestes*/*B. gladiator* (Table 1).

Within *B. aguilari*, there was no sequence divergence (UPD 0.00%) and within most *B. palaestes* it amounts to a moderate level (UPD 0.00–1.31%). However, the *B. palaestes* sample CORBIDI 29695 from Puente San Miguel, La Convención, Cusco, shows somewhat more pronounced distances to other *B. palaestes* (UPD 1.19–1.73%) and a remarkably low distance (UPD 1.79%) to a *B. gladiator* sample (CORBIDI 30241) from lowland rainforest at Kotsimba-Bahuaja, Puno department. Another available *B. palaestes* specimen from a nearby locality (MUBI 17571), also in La Convención, Cusco, shows UPD of 0.79–1.31% to other *B. palaestes*, 1.19–1.31% to all specimens of *B. aguilari*, and 2.38–3.33% to all *B. gladiator*.

Bioacoustics

In the following, we describe the advertisement calls of certain populations of Peruvian species in the *Boana balzani* clade, all of them recorded in the field, plus that of *B. balzani* from Bolivia, and evaluate these with respect to intra-specific variation versus species-specific differences. Numerical parameters of analyzed calls are summarized in Table 2.

Boana aguilari: Advertisement calls were recorded on 6 February 2025, 23:15 h (12.8°C air temperature) at its type locality in the San Alberto valley (10°32'54" S, 75°22'25" W, 2170 m a.s.l.), east of Oxapampa, Pasco department, Peru. Males were calling at the edge of a large artificial pond, hidden in partly submerged vegetation, close to the water surface. The call consists of a long, low-pitched, pulsed note, repeated at irregular intervals (Fig. 2). Pulses within notes are clearly separated, all approximately with the same duration, and separated by inter-pulse intervals of very similar duration. Pulses exhibit an increasing relative amplitude from the beginning to approximately the last third of the call's duration, where call energy reaches its maximum. Numerical parameters of 26 analyzed calls of two males are as follows: call duration (= note duration), 435–628 ms (532.5 ± 57.9 ms); pulses/call, 15–24 (19.9 ± 2.8); pulses/s, 32–44 (39.8 ± 3.7); dominant frequency, 818–1291 Hz (1170 ± 139 Hz); prevalent bandwidth 700–2600 Hz.

Boana palaestes: Advertisement calls were recorded on 21 February 1975, 1:30 h (14.0°C air temperature) at its type locality, southern edge of Tutumbaro, (12°42' S, 73°54' W, 1840 m a.s.l.), 5.4 km by road SW of Ayna, Ayacucho department, Peru. Males were calling at the edge of a low-gradient stream from dense vegetation within disturbed montane forest (DUELLMAN et al. 1997). The call consists of a long, low-pitched, pulsed note, repeated at irregular intervals (Fig. 2). Pulses within notes are clearly separated, all of them approximately with the same duration, and separated by inter-pulse intervals of very similar duration. Pulses exhibit an increasing relative amplitude from the beginning to approximately the middle of the call's duration, where call energy reaches its maximum. Numerical parameters of 12 analyzed calls of one male are as follows: call

duration (= note duration), 370–523 ms (443.5 ± 47.3 ms); pulses/call, 14–22 (18.6 ± 2.6); pulses/s, 35–48 (40.7 ± 3.3); dominant frequency, 1055–1184 Hz (1105 ± 41 Hz); prevalent bandwidth 800–2400 Hz.

A single call of *B. palaestes* recorded on 24 October 2022 (15.0 °C air temperature), ca. 2.7 km SE of Ccentabamba ($12^{\circ}41'34''$ S, $73^{\circ}50'56''$ W, 1680 m a.s.l.), about 11 km air-line distance NE of Tutumbaro, Ayacucho department,

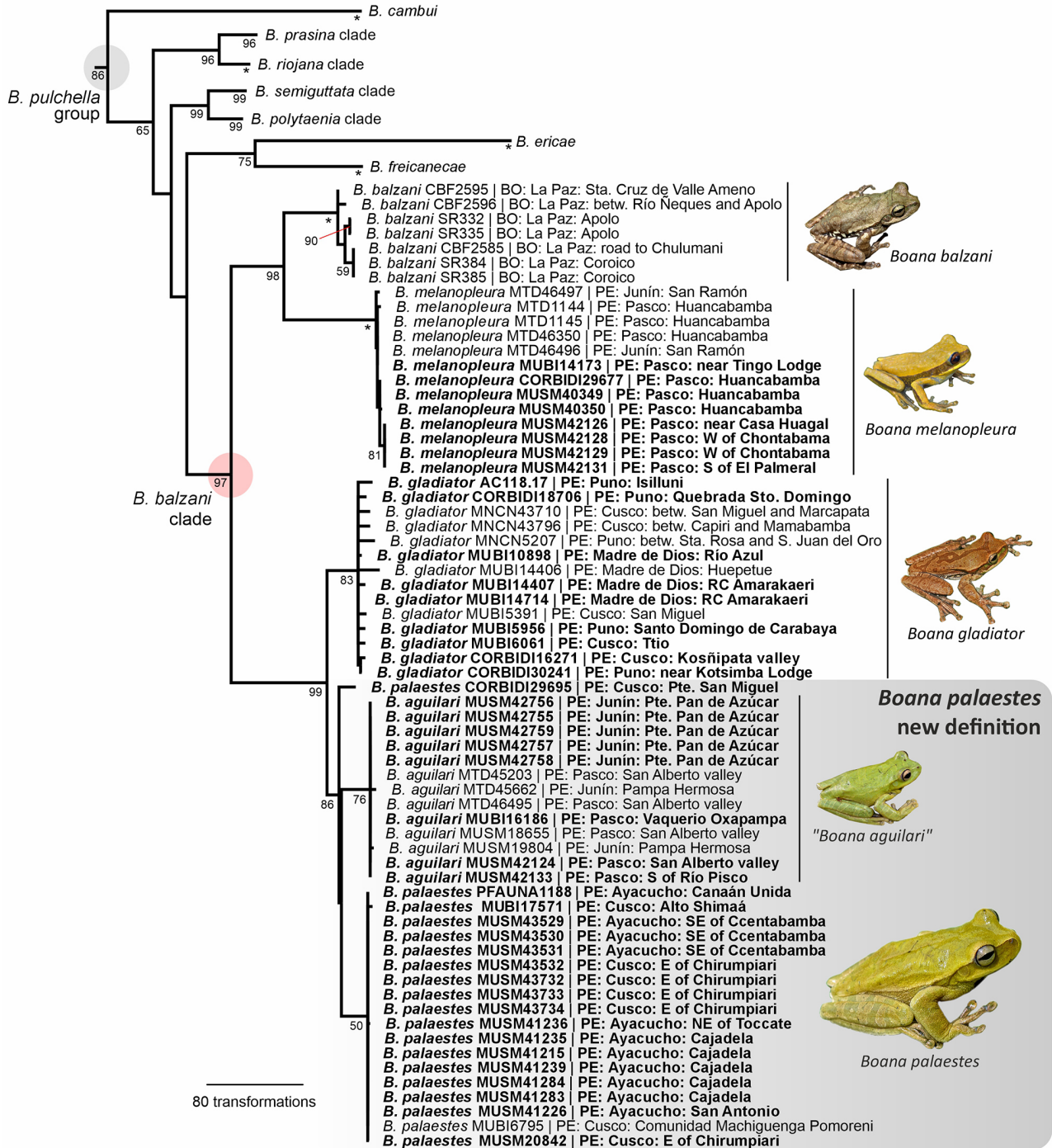


Figure 1. Details of the section of the *Boana balzani* clade and its relationships with other clades in the *B. pulchella* group in the strict consensus of the 816 most parsimonious multigene trees (24353 steps), from the analysis considering gaps as a fifth state. Numbers at nodes are jackknife parsimony values; only values above 50% are shown; asterisks on nodes indicate 100% jackknife. Terminal names in bold indicate samples sequenced for this study. Abbreviations in sample localities are: BO = Bolivia; PE = Peru.

Table 1. Range of uncorrected pairwise distances calculated for the mitochondrial 16S rRNA gene in phylogenetic subclades referring to nominal species in the *Boana balzani* clade in percent (%). The sample CORBIDI 29695 (*B. palaestes*) is listed separately as its relationships to the *B. aguilari* and *B. palaestes* clades are poorly supported in the parsimony tree. Gray-shaded cells refer to intra-subclade distances.

	<i>B. aguilari</i>	<i>B. palaestes</i>	<i>B. gladiator</i>	<i>B. melanopleura</i>	<i>B. balzani</i>	CORBIDI 29695
<i>B. aguilari</i>	0.00					
<i>B. palaestes</i>	1.19–2.36	0.00–1.31				
<i>B. gladiator</i>	2.11–3.56	2.31–3.67	0.00–1.89			
<i>B. melanopleura</i>	4.70–6.63	4.88–7.11	4.81–6.61	0.00–0.39		
<i>B. balzani</i>	3.51–4.51	4.74–5.42	3.79–4.50	3.62–4.86	0.00–0.39	
CORBIDI 29695	1.60–1.73	1.19–1.73	1.79–3.09	4.34–6.19	4.28–4.63	–

Table 2. Numerical parameters of advertisement calls of species/populations in the *Boana balzani* clade recorded in the field. Pulse rate was calculated within calls; * indicates recordings obtained from topotypes; BO = Bolivia; PE = Peru; n.a. = not available.

Species	Locality	Call duration [ms]	Pulses/call	Pulses/s	Dominant frequency [Hz]	Temp. [°C]	Calls analyzed
<i>B. aguilari</i> *	PE: Pasco: San Alberto	435–628 (532.5 ± 57.9)	15–24 (19.9 ± 2.8)	32–44 (39.8 ± 3.7)	818–1291 (1170 ± 139)	12.8	26
<i>B. palaestes</i> *	PE: Ayacucho: Tutumbaro	370–523 (443.5 ± 47.3)	14–22 (18.6 ± 2.6)	35–48 (40.7 ± 3.3)	1055–1184 (1105 ± 41)	14.0	12
<i>B. palaestes</i>	PE: Ayacucho: 11 km NE Tutumbaro	432	22	55–58	1205	15.0	1
<i>B. gladiator</i> *	PE: Cusco: San Miguel	321–479 (408.9 ± 57.9)	42–53 (45.0 ± 4.5)	100–118 (109.0 ± 5.9)	1248–1593 (1431 ± 134)	20.0	7
<i>B. gladiator</i>	PE: Puno: San Juan del Oro	353–556 (457.4 ± 67.0)	50–68 (57.6 ± 4.9)	111–167 (135.8 ± 14.9)	1442–1722 (1608 ± 91)	25.0	21
<i>B. gladiator</i>	PE: Cusco: Kosñipata valley	245–423 (330.9 ± 48.5)	24–37 (31.6 ± 3.2)	95–111 (103.7 ± 5.8)	1104–1740 (1380 ± 185)	n.a.	23
<i>B. melanopleura</i> *	PE: Pasco: Huancabamba	231–320 (287.0 ± 36.5)	7–12 (8.8 ± 2.2)	22–43 (30.8 ± 7.7)	960–1168 (1101 ± 88)	12.4	5
<i>B. melanopleura</i>	PE: Pasco: Chontabamba	203–403 (266.0 ± 54.8)	5–11 (7.1 ± 1.5)	19–52 (33.0 ± 9.9)	937–1497 (1120 ± 172)	14.8	24
<i>B. balzani</i>	BO: La Paz: near Apolo	234–244	10	40–43	1084–1129	n.a.	2

Peru, agrees in character with those reported from the type locality Tutumbaro, except for a slightly higher pulse rate. Its numerical parameters are as follows: call duration (= note duration), 432 ms; pulses/call, 22; pulses/s, 55–58; dominant frequency, 1205 Hz; prevalent bandwidth 900–2400 Hz.

Boana gladiator: Advertisement calls were recorded on 21 February 2006 (20.0 °C air temperature) at its type locality San Miguel (13°24'14.1" S, 70°53'57.7" W, 1155 m a.s.l.), Marcapata valley, Cusco department, Peru. Males were calling from road-side ditches in disturbed rainforest (KÖHLER et al. 2010). The call consists of a long, rapidly pulsed note, repeated at irregular intervals. Pulses are very short (ca. 4–6 ms) and clearly separated. Pulses exhibit an increasing relative amplitude from the beginning to approximately the middle of the call's duration, where call energy reaches its maximum. Inter-pulse intervals tend to be slightly longer at the beginning and at the end of the

call. Numerical parameters of 7 analyzed calls of at least two males are as follows: call duration (= note duration), 321–479 ms (408.9 ± 57.9 ms); pulses/call, 42–53 (45.0 ± 4.5); pulses/s, 100–118 (109.0 ± 5.9); dominant frequency, 1248–1593 Hz (1431 ± 134 Hz); prevalent bandwidth 800–2900 Hz.

Advertisement calls of *B. gladiator* recorded on 12 February 2006 (25 °C air temperature; erroneously given as 20 °C by KÖHLER et al. 2010) near San Juan del Oro (14°11'2.4" S, 69°04'46.2" W, 1097 m a.s.l.), Puno department, Peru, are in agreement with those from the type locality, apart from a slightly higher pulse rate and a somewhat higher number of pulses per call (Fig. 2). Numerical parameters of 21 analyzed calls of two males are as follows: call duration (= note duration), 353–556 ms (457.4 ± 67.0 ms); pulses/call, 50–68 (57.6 ± 4.9); pulses/s, 111–167 (135.8 ± 14.9); dominant frequency, 1442–1722 Hz (1608 ± 91 Hz); prevalent bandwidth 700–3500 Hz.

Advertisement calls of *B. gladiator* recorded in 2021 (air temperature not recorded) at San Pedro (13°03'30" S, 71°32'53" W, 1400 m a.s.l.), Kosñipata valley, close to the border of Parque Nacional Manu, Cusco department, Peru, are in agreement with those reported above, but are slightly

shorter in duration and contain a lower number of pulses per call (Fig. 2). Numerical parameters of 23 analyzed calls of four males are as follows: call duration (= note duration), 245–423 ms (330.9 ± 48.5 ms); pulses/call, 24–37 (31.6 ± 3.2); pulses/s, 95–111 (103.7 ± 5.8); dominant frequency,

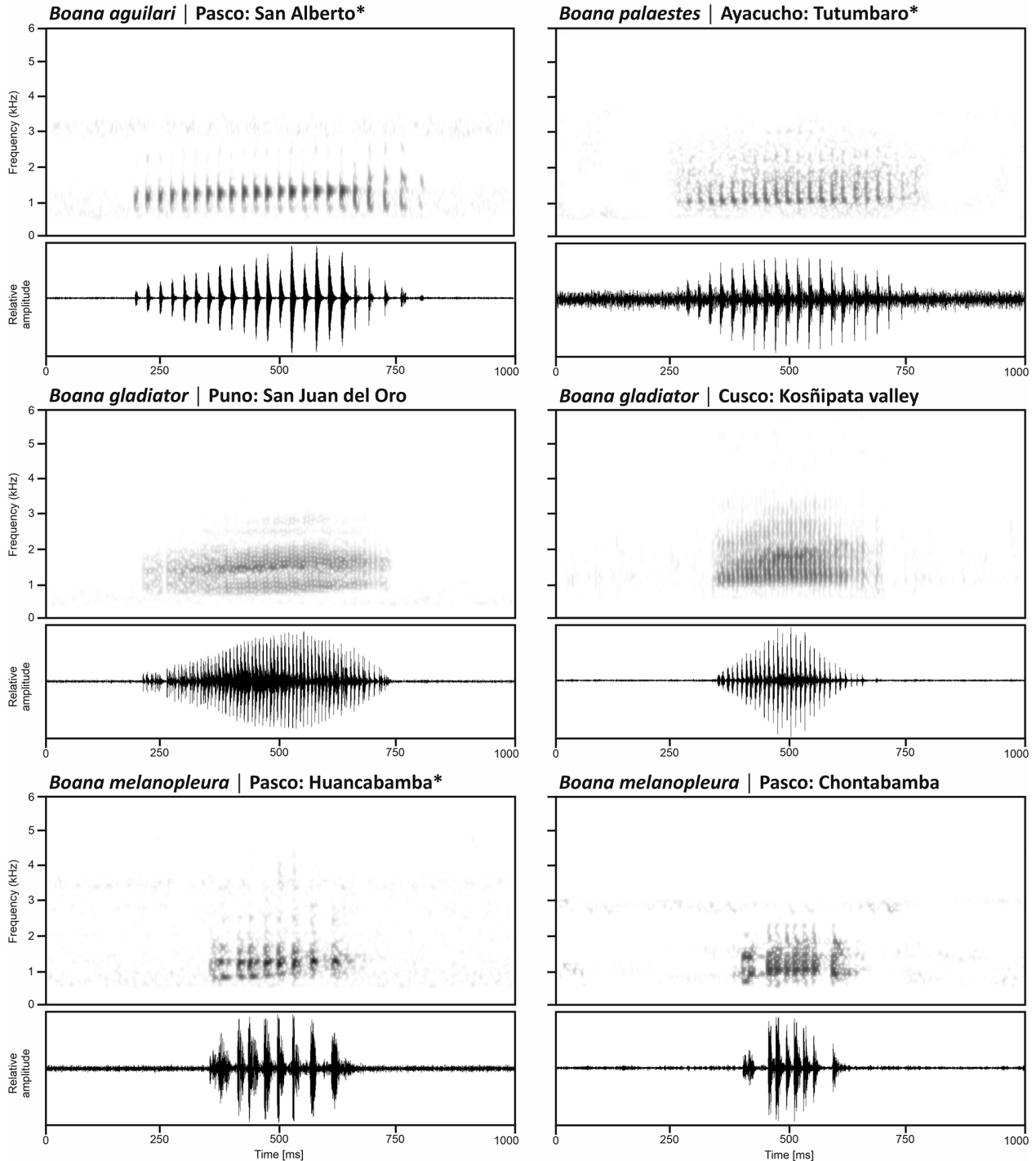


Figure 2. Audiospectrograms and corresponding oscillograms of advertisement calls of Peruvian species/populations in the *Boana balzani* clade, all displayed at the same frequency and time scale (1000 ms). Asterisks behind the given locality indicate calls from topotypical males.

1104–1740 Hz (1380 ± 185 Hz); prevalent bandwidth 900–4700 Hz.

Boana melanopleura: Advertisement calls were recorded on 20 November 2019 (air temperature 12.4°C) at the type locality Huancabamba, Hacienda Yanachaga ($10^{\circ}25'08''\text{S}$, $75^{\circ}31'07''\text{W}$, 1800 m a.s.l.), Pasco department, Peru. Males were calling at a small artificial pond, hidden in vegetation close to the water surface. The call consists of a pulsed note of moderate duration, repeated at irregular intervals (Fig. 2). Pulses within calls are clearly separated and vary in duration from 19–55 ms. Pulse structure within calls is irregular, with initial and terminal pulses being of longer duration, having less call energy and being separated from pulses in the call's center by longer inter-pulse intervals. Numerical parameters for five analyzed calls emitted by two males are as follows: call duration (= note duration), 231–320 ms (287.0 ± 36.5 ms); pulses/call, 7–12 (8.8 ± 2.2); pulses/s, 22–43 (30.8 ± 7.7); dominant frequency, 960–1168 Hz (1101 ± 88 Hz); prevalent bandwidth 600–2800 Hz.

Calls of *B. melanopleura* recorded on 7 February 2025 (air temperature 14.8°C) in a swampy area within the riverbed of the Río Chontabamba, W of Chontabamba ($10^{\circ}36'04''\text{S}$, $75^{\circ}28'47''\text{W}$, 1870 m a.s.l.), Pasco department, Peru, generally agree with calls described above from the type locality (Fig. 2). Equally, an irregular pulse structure within calls is evident. Numerical parameters for 24 analyzed calls emitted by at least four males are as follows: call duration (= note duration), 203–403 ms (266.0 ± 54.8 ms); pulses/call, 5–11 (7.1 ± 1.5); pulses/s, 19–52 (33.0 ± 9.9); dominant frequency, 937–1497 Hz (1120 ± 172 Hz); prevalent bandwidth 700–2800 Hz.

Boana balzani: The advertisement call of *B. balzani* from near Apolo, La Paz department, Bolivia, has been described and figured by KÖHLER et al. (2010). It consists of a pulsed note of moderate duration, with pulses being clearly separated. Numerical parameters for two analyzed calls emitted by one male are as follows: call duration (= note duration), 234–244 ms; pulses/call, 10; pulses/s, 40–43; dominant frequency, 1084–1129 Hz; prevalent bandwidth 300–2100 Hz.

Morphology

Given the diagnostic character 'presence of white spots on flanks' used by LEHR et al. (2010) to distinguish *Boana aguilari* from *B. palaestes*, we investigated this character in 33 specimens of *B. palaestes*. Among the 15 adult specimens of *B. palaestes* deposited in the KU collection (see Appendix), four males have series of creamy-white spots and/or flecks on flanks (KU 163308, 196846, 196848, 196849). Of the 13 adult males of *B. palaestes* in the MUSM collection (see Appendix), all have creamy-white spots on the flanks. These spots are distinctly contrasting in most specimens, but minute, less distinct and faint in MUSM 41239, 41283, and 43732 (Fig. 3). Spots on flanks are completely missing in the juvenile MUSM 41236. In 18 adult *B. aguilari* specimens examined (see Appendix), all exhibit creamy-white spots or flecks on flanks. The metamorph MUSM 24806

(snout–vent length 22.3 mm) lacks any spotting on flanks. In summary, all examined adults of *B. aguilari* exhibit spots and/or flecks on flanks, whereas in *B. palaestes* 61% of all examined adults exhibit spotted flanks.

LEHR et al. (2010) mention the presence of a thin dorso-lateral fold shared by *B. aguilari* and *B. palaestes*. We were able to confirm the presence of such a dorsolateral fold in most specimens of the two nominal species. This character is less obvious in preserved specimens, but even in some living individuals, the folds may be poorly differentiated and barely discernible (Fig. 4).

It is worth mentioning that in both *B. aguilari* and *B. palaestes* predominantly green and predominantly brown color morphs exist (sometimes some intermediate color is present). These green and brown morphs do occur together at both respective type localities of the two nominal taxa. Reproductively active individuals of both nominal taxa exhibit blue or bluish-gray color in the groin, axillae and ventral surfaces of limbs (Fig. 4).

Measurements and proportions in both *B. aguilari* and *B. palaestes* agree with the respective values reported in the original descriptions (LEHR et al. 2010, DUELLMAN et al. 1997) and broadly overlap with each other, as well as with values reported for *B. gladiator* by KÖHLER et al. (2010). Morphometrics are thus not diagnostic, and we therefore refrain from presenting detailed measurements.

Discussion

Phylogeny and sequence divergence

Our phylogenetic results are congruent with recent studies on the *Boana pulchella* group (FAIVOVICH et al. 2021, NAKAMURA et al. 2025) in all highly supported clades. Particularly, they corroborate the monophyly of the *B. balzani* clade, the species status of *B. balzani* and *B. melanopleura*, and the close relationship between *B. aguilari*, *B. gladiator*, and *B. palaestes*. The inclusion of additional specimens of *B. aguilari* and *B. palaestes* from several localities corroborate their monophyly. This close relationship is further supported by the relatively small uncorrected p-distances of 1.19–2.36% (Table 1) between them, corroborating the observations of FAIVOVICH et al. (2021), which were based on a smaller sample size. These authors also reported the relatively small UPDs between the latter two species and *B. gladiator*. All samples of *B. gladiator* are recovered here with high support, and exhibit UPDs of 2.11–3.67% relative to the clade including *B. aguilari* and *B. palaestes*.

Within-clade UPDs of *B. aguilari*/*B. palaestes* are 0.00–2.36%, with higher values largely explainable by geographic distance among respective samples (with exception of sample CORBIDI 29695; see above), and are not indicative of species-level differentiation given the results from bioacoustics and morphology. UPDs within *B. gladiator* amount to higher values (0.00–1.89%) when compared to *B. balzani* and *B. melanopleura* (0.00–0.39%, respectively) and may similarly reflect a considerably larger range covered by the *B. gladiator* samples included in the analysis.

In amphibians, reproductive isolation is in general positively, though imperfectly, correlated with genetic divergence, which, in theory, is proportional to the time since evolutionary divergence (e.g., DUFRESNES et al. 2021). While a genetic distance of 3% in the 16S rRNA sequence fragment has been found to often indicate divergence at the species level (FOUQUET et al. 2007), this value is heuristic and needs to be carefully evaluated and interpreted in each taxon. When analyzed in combination with phylogenetic and phenotypic evidence (vocalization, morphology), distance values below 3% have been found between lineages differentiated at the species level in some groups of anurans (e.g., VENCES et al. 2012, PEREYRA et al. 2021) while values distinctly higher than 3% characterize species in other groups (e.g., VENCES et al. 2011, KÖHLER et al. 2024). From this perspective, the lineages of *B. gladiator* and *B. aguilari*/*B. palaestes* can be considered to be within the ‘grey zone of speciation’ (ROUX et al. 2016) where genetic distances alone cannot provide strong arguments for species delimitation. We can, however, conclude that low UPDs < 3% would not contradict treating *B. aguilari* and *B. palaestes* as conspecific.

Bioacoustics

Our analysis of advertisement calls of species in the *Boana balzani* clade revealed that all consist of distinctly pulsed notes of moderate to long duration, and exhibit rather similar durations and dominant frequency ranges (Table 2). Although intraspecific variation in call parameters is recognizable to a moderate extent (Table 2), qualitative and quantitative call characteristics are in concordance with identified lineages in most cases and allow for their identification by bioacoustics alone. Calls of *B. melanopleura* are distinguished from calls of other Peruvian species in the clade by their irregular pulse structure, considerable variation of pulse duration within calls, and the lowest number of pulses per call (5–12 versus 14–68 in other nominal species). Calls of *B. gladiator* are distinguished from those of all other nominal species in the clade by a distinctly higher pulse rate within calls (95–167 pulses/s versus 19–58 pulses/s in other nominal species) and the highest maximum number of pulses per call (68 versus 5–24 in other nominal species). Calls of *B. balzani* mainly differ from those of *B. aguilari*, *B. gladiator*, and *B. palaestes* by short-



Figure 3. Adult male *Boana palaestes* in life, bodies in lateral or dorsolateral views, exemplifying variation in flank coloration: (A) MUSEM 43529, (B) MUSEM 43530, and (C) MUSEM 43531, all three from near Ccentabamba (11 km NE of the type locality Tulumbaro), Ayacucho; (D) MUSEM 43532, from near Chirumpiari, La Convención, Cusco; (E) MUSEM 41239 and (F) MUSEM 41283, both from Cajadela, Ayacucho.

er duration, and from those of *B. melanopleura* by a very regular pulse structure (versus irregular). In summary, the qualitative and quantitative call differences observed are

beyond those that could be considered intraspecific call variation (see KÖHLER et al. 2017) and support the specific distinctness of *B. balzani*, *B. gladiator*, and *B. melanopleu-*

Boana aguilari



Boana palaestes

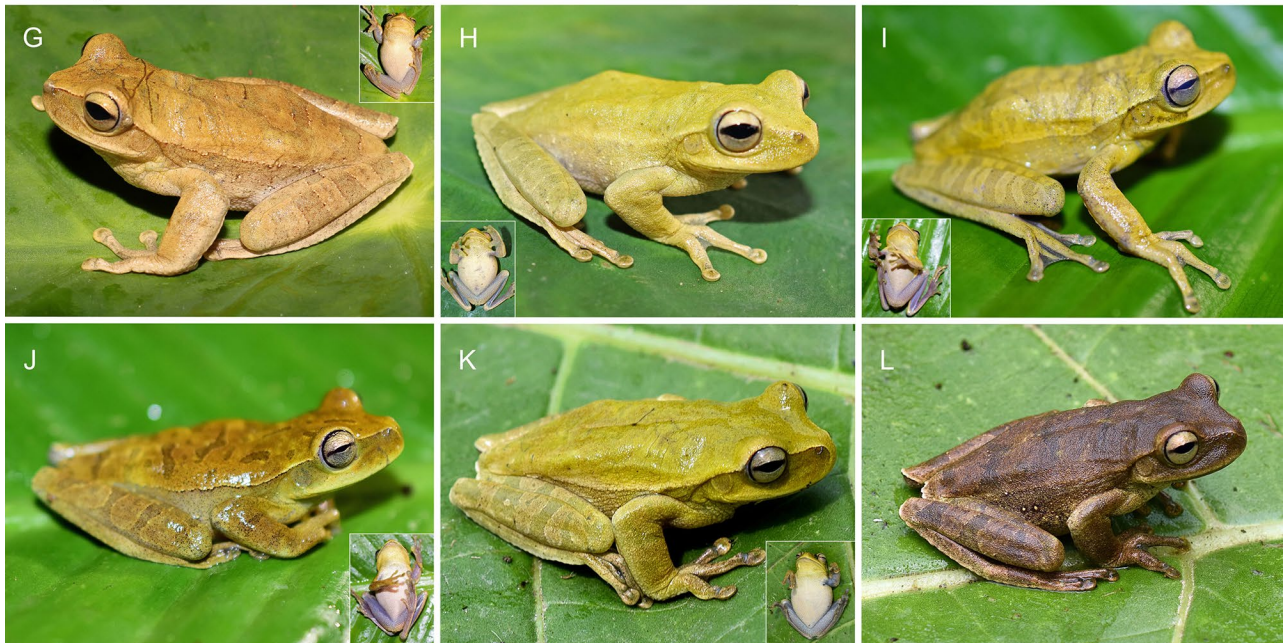


Figure 4. Adult males of the nominal taxa *Boana aguilari* and *B. palaestes* in life in dorsolateral views, exemplifying variation in color pattern (in some cases small inset photos show ventral surfaces): (A) MUSM 18656 (holotype) from San Alberto, Pasco; (B) MUSM 42124 and (C) MUSM 42125, topotypes, both from San Alberto, Pasco; (D) MUSM 42133 from Río Pisco, Pasco; (E) MUSM 31124, San Juan de Miraflores, and (F) MUSM 32725, Ayte, both in Pui Pui Protected Forest, Junín; (G) MUSM 41235 and (H) MUSM 41215 both from Cajadela, Ayacucho; (I) MUSM 43734 and (J) MUSM 43532 both from near Chirumpiari, Cusco; (K) MUSM 43530 and (L) MUSM 43531 both from near Ccentabamba (11 km NE of the type locality Tutumbaro), Ayacucho.

ra. In contrast, calls recorded from males of *B. aguilari* and *B. palaestes*, including respective topotypes, are identical in qualitative characters and broadly overlap in all quantitative parameters (Fig. 2, Table 2), despite ca. 300 km distance between their type localities. Thus, bioacoustic analyses do not identify two species-specific advertisement calls among populations of *B. aguilari* and *B. palaestes*.

It is worth mentioning that both KÖHLER et al. (2010) and LEHR et al. (2010) relied on the *B. palaestes* call description by DUELLMAN et al. (1997) when they diagnosed *B. gladiator* and *B. aguilari*, respectively. As demonstrated by FAIVOVICH et al. (2021: suppl. data 6), the call description by DUELLMAN et al. (1997) was erroneously based on vocalization of a centrolenid frog calling in the background in the recording from Tutumbaro, consequently resulting in pronounced differences of the calls to those of other *Boana*. Actually, calls of *B. palaestes* are much more similar to those of *B. gladiator* and identical to those of *B. aguilari* (see above).

When LEHR et al. (2010) described the calls of *B. aguilari* and *B. melanopleura*, they used recordings obtained in captivity by K.-H. JUNGFER, with both species contained in the same tank. These authors emphasized the surprisingly sim-

ilar call characteristics of both species. Although call duration might be similar, as shown by our analyses, the calls of both nominal taxa differ in the number of pulses per call and most importantly in pulse structure (Fig. 2, Table 2), and calls of the two species are clearly distinguishable by the human ear in the field. This leads us to speculate that the actual amplitude modulation of both calls recorded in captivity was masked by echo effects, i.e. sound reflections from the tank walls, as indicated by the poor quality of the spectrograms and oscillograms with barely recognizable separated pulses (LEHR et al. 2010: Figs 4 and 5), or/and that perhaps the same species may have been recorded twice.

Morphology

In the diagnosis of *Boana aguilari*, LEHR et al. (2010) used the presence of cream/white spots on flanks in this species to distinguish it from *B. palaestes*, which lacks those spots according to its original description (DUELLMAN et al. 1997). However, KÖHLER et al. (2010) mentioned that in *B. palaestes* the flanks are uniformly colored, sometimes with small cream spots. Our analysis of the type series of

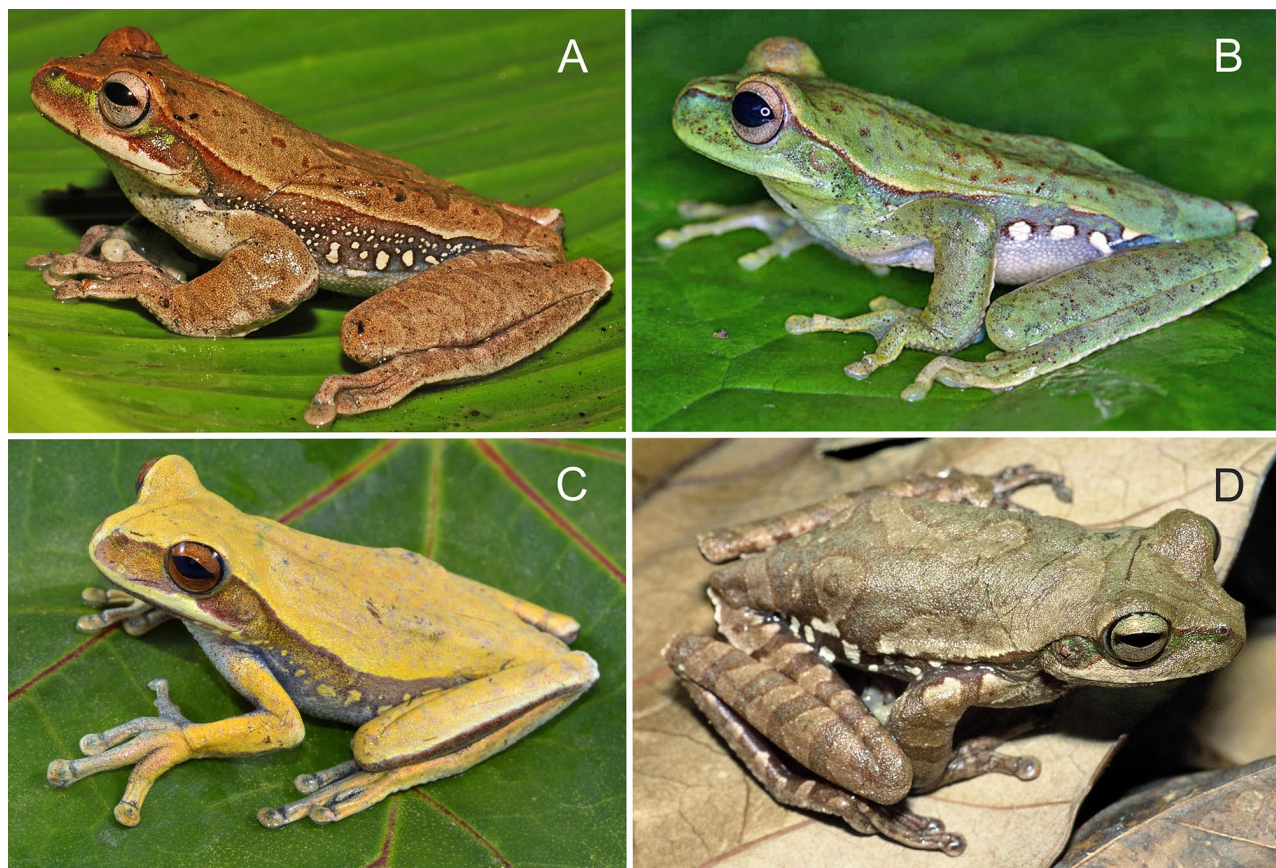


Figure 5. Individuals of other species in the *Boana balzani* clade in life for comparison: (A) *B. gladiator* (MUBI 5391, holotype), from San Miguel, Marcapata valley, Cusco, Peru; (B) *B. gladiator* (MUBI 5956, paratype), from Santo Domingo de Carabaya, Puno, Peru; (C) *B. melanopleura* (MUSM 42129), from near Chontabamba, Pasco, Peru; (D) *B. balzani* (voucher number unknown), from near Apolo, La Paz, Bolivia (courtesy of I. DE LA RIVA).

B. palaestes shows that ca. 21% of these specimens have spots on flanks. When combined with recently collected specimens, a total of 61% of adult *B. palaestes* specimens exhibit more or less distinct spots or flecks on flanks, corroborating the observation by KÖHLER et al. (2010) and revealing plasticity in this character. Conversely, LEHR et al. (2010) did not mention variation in this trait for *B. aguilari*. Our examination of part of the *B. aguilari* original type series as well as recently collected specimens confirmed this observation, with all adult specimens exhibiting spotted flanks. However, spots on flanks are absent in a *B. aguilari* metamorph (as well as in a juvenile *B. palaestes*), arguing for an ontogenetic change in this pattern. Given the variation of this trait found in adult *B. palaestes*, we conclude that the presence/absence of white spots or flecks on flanks is not a character distinguishing *B. aguilari* and *B. palaestes*.

Our measurements of adult males of *B. palaestes* agree with the original measurements provided by DUELLMAN et al. (1997) and also overlap with the original measurements of *B. aguilari* provided by LEHR et al. (2010), indicating that both taxa do not differ in the studied morphological characters.

Boana aguilari and *B. gladiator* were described almost concomitantly, and therefore were not compared to each other in their original publications. Their measurements broadly overlap, and the color pattern might be rather similar as well. However, while *B. aguilari* exhibits series of white spots on flanks, the markings on the flanks of *B. gladiator* are mostly larger, representing white blotches, in many cases surrounded by small white flecks as well (Fig. 5; KÖHLER et al. 2010). Most *B. gladiator* individuals exhibit a sharply contrasting color border between the dorsum and darker flanks (Fig. 5). However, such a pattern was also observed in few individuals of *B. aguilari* and *B. palaestes*. Although apparently less contrasting in the two latter taxa, we do not consider it a diagnostic character at the current state of knowledge. The same applies to the presence or absence of dorsolateral folds. Although we did not observe dorsolateral folds in *B. gladiator* individuals, such folds seem to vary greatly in distinctness among individuals of *B. aguilari* and *B. palaestes*. More comprehensive studies are needed to confirm or reject mentioned morphological characters as being diagnostic.

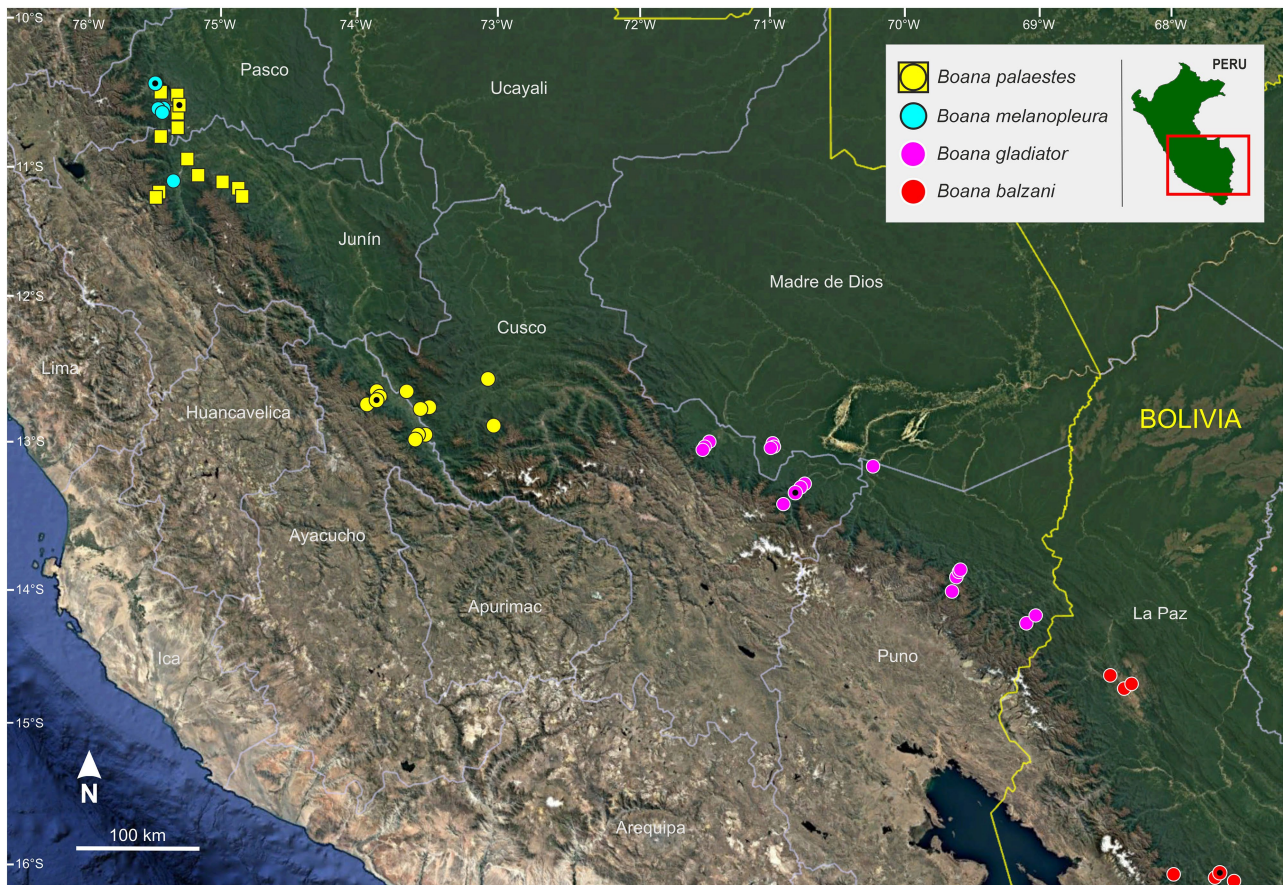


Figure 6. Satellite image (from Google Earth) of central and southern Peru and adjacent Bolivia, showing the updated known distribution of species in the *Boana balzani* clade as derived from own sampling and literature sources. Yellow squares refer to populations formerly referred to as *B. aguilari*. Black dots within colored symbols indicate the respective type localities. Pale lines indicate political borders of Peruvian and Bolivian departments.

LEHR et al. (2011) revisited the external morphology of larvae of species of the *Boana balzani* clade, but for *B. palaestes* they relied on information provided by DUELLMAN et al. (1997) and differences found between *B. aguilari* and *B. palaestes* tadpoles were restricted to coloration subtleties. Given that no photos in life or in preservative were provided, such subtle differences could even reflect different interpretations of characters by different groups of authors. Unfortunately, tadpoles of the *B. balzani* clade mentioned by DUELLMAN et al. (1997), and housed at KU (lots: KU 164051, *B. palaestes*; KU 164052–164054, *B. balzani*, but given their collecting locality probably representing *B. gladiator*) are in poor condition and their morphology and coloration cannot be reliably assessed.

Taxonomic conclusions

Given our results, namely monophyly, low genetic divergence, lack of diagnostic morphological characters, and lack of species-specific differences in the advertisement call, we conclude that the nominal taxa *Boana aguilari* and *B. palaestes* are conspecific. Consequently, we consider *Hypsiboas aguilari* LEHR, FAIVOVICH & JUNGFER, 2010 as a junior synonym of *Boana palaestes* (DUELLMAN, DE LA RIVA & WILD, 1997). A complete synonymy and a chresonymy for *B. palaestes* are provided in Supplementary data.

Moreover, we corroborate the status of *Boana balzani*, *B. melanopleura*, and *B. gladiator* as distinct species. Despite the close relationship between *B. gladiator* and *B. palaestes*, the two species are clearly distinguished by their advertisement calls (and possibly subtle differences in morphology).

Species' ranges

Taking into account our updated taxonomy in the *Boana balzani* clade and additional samples included in our analysis, the following geographical ranges for the four recognized species are revealed (Fig. 6): (1) For *B. palaestes*, two major, discontinuous areas are evident, with one along the eastern Andean slopes in central Peru (Junín and Pasco departments), including the Pui Pui Protected Forest (LEHR & MORAVEC 2024), and the other in the Cusco and Ayacucho departments, with about 190 km airline distance between the closest localities from the two areas, that we interpret as likely representing a collection gap. Elevational distribution ranges from 682 to 2280 m a.s.l.; (2) *B. gladiator* occurs along the Andean slopes and adjacent lowlands in the Cusco, Madre de Dios, and Puno departments. Some localities are rather close to the Bolivian border and its occurrence there is expected. There is a gap of ca. 160 km airline distance to the closest known locality of *B. palaestes*. Collection activities in a potential contact zone of these closely related species are recommended. The elevational distribution of *B. gladiator* ranges from 450 to 1980 m a.s.l.; (3) *B. melanopleura* occurs along the eastern

Andean slopes in central Peru (Junín and Pasco departments) between 1800–2070 m a.s.l., in close parapatry with *B. palaestes*. Remarkably, as already mentioned by LEHR et al. (2010) and here confirmed, *B. melanopleura* and *B. palaestes* do not seem to occur in sympatry, although some respective localities are very close to each other (< 10 km airline distance); (4) As far as is known, *B. balzani* is restricted to the Yungas de La Paz region in Bolivia, where it occurs between ca. 1150–1700 m a.s.l., partly in sympatry with *B. callipleura* (*B. riojana* clade; KÖHLER et al. 2006, 2010). So far, there are no confirmed records for Peru (former Peruvian records refer to *B. gladiator*), and it may likely represent a Bolivian endemic.

Acknowledgements

We are grateful to the Servicio Nacional Forestal y de Fauna Silvestre (SERFOR) for issuing necessary scientific permits (RGD 024-2017-SERFOR-DGGSPFFS, RGD 434-2017-SERFOR-DGGSPFFS, RGD 071-2020-MINAGRI-SERFOR-DGGSPFFS, RGD D000067-2021-MINAGRI-SERFOR-DGGSPFFS, RGD D000012-2022-MIDAGRI-SERFOR-DGGSPFFS-DGSPFS, RGD D000017-2024-MIDAGRI-SERFOR-DGGSPFFS-DGSPFS-JLR, and 00063-2018-GRA/GG-GRDE-DRAA-DFFS-D). Animal care protocols for fieldwork by AC are IACUC-21-107 and IACUC-24-024. RAFAEL BROWN, RICHARD GLOR, and ANA P. MOTTA (KU) allowed access to specimens under their care. ANTOINE FOUQUET kindly shared some sequences with us, IGNACIO DE LA RIVA provided the photo of *B. balzani* and JOSÉ M. PADIAL call recordings of *B. gladiator*. MAURA FERNÁNDEZ, KIMBERLY NACCHA and JUAN GAMBOA kindly supported fieldwork in Ayacucho department. Fieldwork of JK, ECU and FG in 2025 was supported by BIOPAT e.V. and Gondwanan Conservation and Research e.V. PDPP was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil (proc. # 2018/18473-6; #2018/15425-0; #2021/13764-5) and by the Smithsonian Institution with a Peter Buck postdoctoral fellowship. Fieldwork of AC was supported by NSF DEB 2003497 and the E. O. Wilson Biodiversity Foundation. JF thanks FAPESP (proc. #2018/15425-0 and CEPID #2021/10639-5), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 346/2019 and 059/2021), and CONICET (PIP 2800).

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Appendix

List of specimens examined for this study. Asterisks indicate holotypes.

Boana aguilari: Peru: Pasco department: Oxapampa: San Alberto, MUSM 18654–18655, MUSM 18656*, MUSM 42124–42125 (five adult males), MUSM 24533 (juvenile), MUSM 24534 (adult female); Llamaquizú, MUSM 19956 (adult male); S of Rio Pisco, MUSM 42133 (adult male); road from Oxapampa to Yaupi, MUSM 19957 (adult female); Daniel Alcides Carrión: Tingo, MUSM 23557–23558 (two adult females); Chontabamba: Maria Teresa, MUSM 24806 (metamorph); Junín department: Chanchamayo: Pozo de Pte. Pan de Azúcar, MUSM 42755–42759 (five adult males); Tarma province, MUSM 19804–19805 (two adult females).

Boana palaestes: Peru: Ayacucho department: Tutumbaro, Rio Piene, KU 163305*, KU 163306–163308, KU 163310 (five adult males), KU 163311–163314 (four juveniles); Tutumbaro, San José, Río Santa Rosa, KU 196844–196853 (ten adult males); 11 km NE of Tutumbaro, MUSM 43529–43531 (three adult males); Cajadela, MUSM 41215, MUSM 41235, MUSM 41239, MUSM 41283–41284 (five adult males); between Toccate and Cajadela, MUSM 41236 (juvenile); San Antonio, MUSM 41226 (adult male); Cusco department: La Convención: ascent from Chirumpiari to Cielo Punku, MUSM 43532, MUSM 43732–43734 (four adult males).

Supplementary data

The following data are available online:

Synonymy and chresonymy for *Boana palaestes* (DUELLMAN, DE LA RIVA & WILD, 1997).