COMPARISON OF MORPHOLOGY AND CALLS OF TWO CRYPTIC SPECIES OF *PHYSALAEMUS* (ANURA: LEIUPERIDAE)

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ABSTRACT: We analyzed and described quantitative differences in morphology and calls of *Physalaemus petersi* and *P. freibergi*, both members of the monophyletic *Physalaemus pustulosus* species group. We found significant differences between the two species in both morphometric and call parameters. *Physalaemus petersi* has proportionately longer legs and a narrower dorsum and head than *P. freibergi*. The calls of *P. petersi* are higher in frequency and longer than *P. freibergi*. Discriminant Function Analysis (DFA) of morphometric variables correctly classified 76.7–87.4% of individuals to species. DFA of call variables correctly classified 96.8–100.0% of males to species. *Physalaemus petersi* is found north of the Río Marañon and Río Amazonas in eastern Ecuador, northeastern Peru, and southeastern Colombia; *P. freibergi* is found south of these rivers in Amazonian Brazil, southeastern Peru, and Amazonian Bolivia. Calls and geographic locations are the most reliable means of identifying these species in the field.

Key words: Advertisement call; Amazon Basin; Anura; Cryptic species; Leiuperidae; Morphology; Natural history; *Physalaemus freibergi*; *Physalaemus petersi*

LOWLAND tropical rainforests are generally thought to have high alpha (single site) species diversity, but low beta (among site) diversity, relative to topographically more complex montane cloud forests (Duellman, 1999). In South America, for example, this difference is reflected by larger ranges (and less among site variation in species composition) of anurans in lowland Amazonia than in the tropical Andes (IUCN, 2006). These biogeographic patterns are largely based on the ranges of species defined using only morphological characters. Genetic and call data, however, reveal that often, morphologically-defined species are actually species complexes consisting of multiple, cryptic species (Angulo and Reichle, 2008; Angulo et al., 2003; Ron et al., 2004, 2005, 2006). Cryptic species have been defined as "two or more distinct species that are erroneously classified (and hidden) under one species name" (Bickford et al., 2007). Thus, what were once considered widespread Amazonian species may often be species complexes consisting of several morphologically similar, yet geographically restricted species. Gamma (total) and beta diversity of Amazonian frogs, therefore, is likely underestimated.

Two such cryptic Amazonian frog species are *Physalaemus freibergi* and *P. petersi*. *Physalaemus freibergi* was placed in the synonymy of *P. petersi* by Cannatella and Duellman (1984) based on morphology and was then subsequently resurrected from synonymy by Cannatella et al. (1998) based on limited molecular and call data. A recent phylogeographic analysis of *P. petersi* and *P. freibergi* (including samples from near the type localities of these species) indicates that each species is a monophyletic group separated from each other by 4.64% mean corrected sequence divergence at the 12S, 16S, and intervening valine tRNA genes (Funk et al., 2007). The goals of the current study were to: (1) provide a comparison of the performance of acoustic and morphometric data for assigning individuals to species; (2) describe morphological and call differences between *P. petersi* and *P. freibergi*; and (3) determine the geographic distributions of each species.

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METHODS

Morphological terminology and abbreviations follow Lynch and Duellman (1997). Sex was determined by the presence of vocal sac folds, nuptial pads, and/or gonadal inspection. Snout–vent length is abbreviated as SVL throughout. Examined specimens (listed in Appendix I) are housed in the California Academy of Sciences (CAS); Museo de Historia Natural de la Universidad Nacional Mayor de San Marcos, Peru (MUSM); Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ); Museum of Comparative Zoology, Harvard University (MCZ); Museum of Vertebrate Zoology, University of California Berkeley (MVZ); Natural History Museum, University of Kansas (KU); Sam Noble Oklahoma Museum of Natural History, University of Oklahoma (OMNH); Smithsonian National Museum of Natural History, Washington, DC (USNM); Texas Cooperative Wildlife Collection, Texas A&M University (TCWC); and Texas Natural History Collection, Texas Memorial Museum, University of Texas at Austin (TNHC).

Recordings were made with a Sennheiser SE66 microphone (frequency response 40–20,000 Hz) or a Sony ECM-MS 907 microphone (frequency response 100–15,000 Hz), a Sony WM-D6C professional analog tape recorder (frequency response 40–15,000 Hz), and metal cassette tapes. Calls were digitized using SIGNAL (Engineering Design, Belmont, MA) at a sampling rate of 25 kHz and a frequency grid resolution (FTP) of 8192 points. Digitized calls were then analyzed using batch processing in SIGNAL. Only one randomly chosen call from each male was analyzed to avoid pseudo-replication. Batch processing enforces a degree of standardization that is sometimes lost when calls are analyzed individually. Calls were examined visually prior to analysis to make sure they had a high signal to noise ratio (i.e., no interference from calls from other males). We measured 14 call parameters: call duration (duration of the entire call); dominant frequency (dominant frequency of the entire call); whine duration (duration of the whine, the main frequency modulated component of the call); whine dominant frequency; maximum frequency (maximum frequency of the whine's fundamental frequency); initial frequency (initial frequency of the whine's fundamental frequency); time to half frequency (time from the start of the whine to its mid-frequency); final frequency (final frequency of the whine); rise time (time from the beginning of the whine to the maximum amplitude of the whine); half rise time (time from the beginning of the whine to half the maximum amplitude); fall time (time from the maximum amplitude of the whine to the end of the whine); half fall time (time from the maximum amplitude of the whine to half the amplitude); prefix duration (duration of the prefix, a short note preceding the whine); and dominant frequency of the prefix (see Fig. 1, Ryan and Rand, 2003). Original recordings are deposited in the audio archive of the Texas Memorial Museum, University of Texas at Austin.

All well-preserved specimens (Simmons, 2002; Table 1) were measured for the following morphometric variables: (1) SVL; (2) dorsal width; (3) tibia length; (4) femur length; (5) arm length; (6) head length; (7) head width; (8) eye–nostril distance; (9) tarsus length; and (10) tubercle–heel length. Vari-
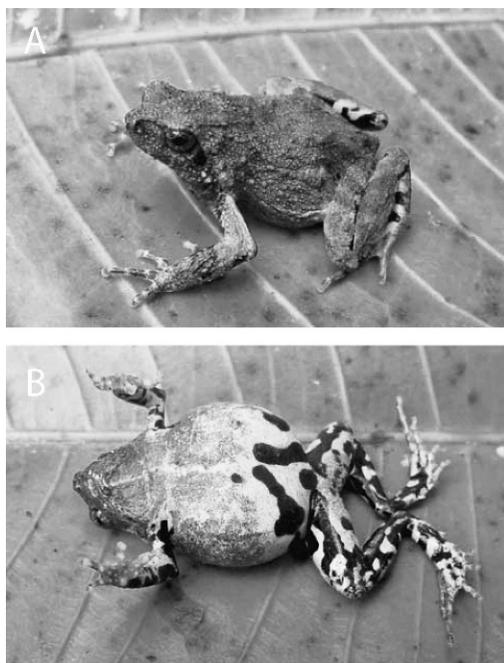

FIG. 1.—Dorsolateral (A) and ventral (B) views of an adult male *Physalaemus petersi*, QCAZ 28584 (from La Selva Hostería, Provincia Sucumbíos, Ecuador).

TABLE 1.—Descriptive statistics for morphometric measurements of *Physalaemus petersi* and *P. freibergi*. Mean ± SD are given with range below. Abbreviations are n = number of specimens; SVL = snout-vent length; DW = dorsal width; TL = tibia length; FL = femur length; AL = arm length; HL = head length; HW = head width; EN = eye-nostril distance; TSL = tarsus length; THL = tubercle-heel length. All measurements are in mm.

| Measurement | Males | | Females | |
	P. petersi	*P. freibergi*	*P. petersi*	*P. freibergi*
n	127	79	60	45
SVL	26.8 ± 2.7	27.8 ± 2.9	30.9 ± 3.4	31.6 ± 3.8
	20.6–31.3	23.5–35.7	25.2–39.1	25.0–39.1
DW	10.4 ± 1.3	11.2 ± 1.6	11.7 ± 1.4	12.2 ± 1.7
	7.9–13.3	8.6–18.5	9.6–15.0	9.1–16.0
TL	14.2 ± 1.6	14.1 ± 1.3	16.0 ± 2.1	15.7 ± 1.6
	10.9–17.3	12.0–16.9	12.2–20.0	12.7–18.9
FL	12.8 ± 1.2	13.5 ± 1.5	15.1 ± 1.8	15.3 ± 1.7
	10.1–15.3	10.9–17.6	11.3–19.1	12.3–18.7
AL	7.5 ± 0.9	7.3 ± 1.1	8.6 ± 1.1	8.4 ± 1.2
	5.2–9.3	5.0–10.2	6.2–10.8	5.7–10.7
HL	8.7 ± 1.0	9.1 ± 0.9	9.6 ± 0.9	9.8 ± 0.9
	6.7–10.8	7.3–11.8	7.6–11.3	8.0–11.8
HW	7.9 ± 0.7	8.7 ± 0.9	8.6 ± 0.9	9.0 ± 0.9
	6.2–9.7	6.9–10.9	7.1–11.2	7.1–10.7
EN	2.3 ± 0.2	2.3 ± 0.3	2.5 ± 0.3	2.5 ± 0.3
	1.7–3.0	1.9–3.3	1.8–3.1	1.8–3.0
TSL	7.5 ± 0.8	7.3 ± 0.6	8.4 ± 1.0	8.1 ± 0.7
	5.8–9.4	6.1–8.6	6.5–10.5	6.1–9.3
THL	5.4 ± 0.6	5.1 ± 0.5	5.9 ± 0.7	5.6 ± 0.6
	4.0–6.7	4.1–6.1	4.4–7.3	4.1–6.7

ables 1–8 were measured according to the methodology described in Duellman (1970) and Ron et al. (2004) with Fowler digital calipers (accurate to the nearest 0.01 mm) from specimens fixed in 10% formalin and preserved in 70% ethanol. Tarsus length was measured from the proximal end of the inner metatarsal tubercle to the heel; tubercle-heel length was measured from the distal end of the inner tarsal tubercle to the heel. These two morphometric variables were measured because of previous reports of variation in the relative position of the tarsal tubercle between *Physalaemus petersi* and *P. freibergi* (Cannatella and Duellman, 1984; Donoso-Barros, 1969; Lynch, 1970). In comparisons of SVL among sites, only sites with more than one specimen were included.

Discriminant Function Analysis (DFA) was used to assign sites without known haplotypes (i.e., sites not included in the Funk et al., 2007, phylogeographic analysis) to either the *P. petersi* or *P. freibergi* clade based on morphometric or call data. First, a discriminant function was developed only using morphometric or call data from sites included in the Funk et al. (2007) phylogeographic study, and therefore known to either be part of the *P. petersi* or *P. freibergi* clade. Then, the discriminant function was used to calculate the mean posterior probability of assignment to *P. petersi* or *P. freibergi* for sites without known haplotypes. We then grouped each site to the species with the highest mean posterior probability. This analysis was performed separately for morphometric and call data. After all sites were assigned to species, Principal Components Analysis (PCA) and DFA were performed using data from all sites to assess the degree of call and morphometric differentiation between the two species. To visualize variation in shape independent of "size", Principal Components Analysis was applied to residuals of the linear regressions between the other nine measured variables and SVL (Vitt et al., 2000). DFA was performed using "raw" morphometric data because the goal of the DFA was to determine the utility of all morphometric variables, including SVL, for distinguishing *P. petersi* and *P. freibergi*. All statistical analyses were performed using Minitab 15.

SYSTEMATIC ACCOUNTS

Physalaemus petersi (*Jiménez de la Espada*)

Engystomops petersi Jiménez de la Espada, 1872; type locality

Eupemphix paraensis Müller, 1923; type locality "Peixeboi (ander Bragançabahn), Staat Pará, Nord-Brasilien."

Eupemphix schereri Myers, 1942; holotype CAS-SU 6317 from "Pevas, at the mouth of the Ampiyacu River, Amazonian Peru," Departamento Loreto.

Physalaemus petersi Lynch, 1970

Engystomops petersi Nascimento, Caramaschi, and Cruz, 2005

Diagnosis.—A member of the genus *Physalaemus* and the *P. pustulosus* group, sensu Cannatella and Duellman (1984) and Cannatella et al. (1998); see Remarks. Assignment to the *P. pustulosus* group is based on the presence of four synapomorphies (Cannatella et al., 1998): (1) presence of flank glands; (2) presence of paratoid glands; (3) warty skin; and (4) dentigerous process of the vomer thin and spikelike.

Physalaemus petersi (Fig. 1) is characterized by: (1) mean SVL 26.8 mm in males (range 20.6–31.3; $n = 127$), 30.9 mm in females (range 25.2–39.1; $n = 60$); (2) skin on dorsum bearing small tubercles with scattered larger tubercles, sometimes forming diverging rows in occipital and scapular regions; (3) snout protruding with anterior margin of lip at a position posterior to the nostrils; (4) vomerine teeth and odontophores absent; (5) maxillary teeth absent; (6) paratoid glands present; (7) flank glands present; (8) tarsal tubercle present; (9) nuptial pads present; (10) Finger I longer than II; (11) tympanic annulus usually well-defined at least ventrally, tympanum smooth; (12) dentigerous process of the vomer thin and spikelike.

The presence of a tarsal tubercle and absence of maxillary teeth immediately distinguishes *P. petersi* from all trans-Andean species in the *Physalaemus pustulosus* species group (clade name Duovox sensu Ron et al., 2006), which includes *P. pustulatus*, *P. guayaco*, *P. montubio*, *P. coloradorum*, and *P. randi*. These species in the lowlands of western Ecuador and northwestern Peru also have Finger I shorter than or equal in length to II (Finger I longer than II in *P. petersi*). *P. pustulosus* differs from *P. petersi* by having a more elongate gland on the flank, a snout with the anterior margin of the lip at the level of the posterior margin of the nostrils, a tuberculate tympanic membrane, and a pattern of tubercles on the dorsum consisting of randomly scattered large and small tubercles or the large tubercles forming longitudinal rows or chevrons. Additionally, *P. pustulosus* lacks the large black inguinal spots characteristic of *P. petersi* and *P. freibergi*. *Physalaemus petersi* differs from *P. freibergi* in advertisement calls and morphology. Mean call duration is significantly longer in *P. petersi* (mean = 253.6 ms, SD = 63.8 ms, $n = 73$; Fig. 2; Table 2) than *P. freibergi* (mean = 116.1 ms, SD = 29.7 ms, $n = 31$, $t = 14.98$, df = 101, $P < 0.001$; Fig. 3). Mean call frequency is significantly higher in *P. petersi* (mean = 591 Hz, SD = 179 Hz, $n = 73$) than *P. freibergi* (mean = 478 Hz, SD = 155 Hz, $n = 31$, $t = 3.21$, df = 64, $P = 0.002$). *Physalaemus petersi* also has proportionately longer legs and a narrower dorsum and head than *P. freibergi* (see Morphometric comparisons in *P. freibergi* account).

Variation.—Dorsal coloration of preserved specimens varies in *P. petersi* (Fig. 4). Most of the variation is in: (1) number and distribution of tubercles; (2) presence or absence of tubercles forming rows in the occipital and scapular regions; (3) hue of the background coloration, varying from gray to dark grey or dark brown; and (4) presence or absence of pale snouts and flanks (referred to as the "pale morph" by Cannatella and Duellman, 1984). In *P. petersi*, significantly more females exhibit the pale morph (39.5%, $n = 60$) than males (2.4%, $n = 127$, Fisher's exact test, $P < 0.001$).

Ventral surfaces of all preserved specimens have a cream or white background with dark markings on the abdomen and gray chests and throats suffused with white flecks (Fig. 1). The color of the markings on the abdomen is generally dark gray to black. Size and number of these dark marks increases toward the groin. The amount of white flecking increases towards the posterior of the chest where it blends in with the cream abdomen. In some individuals, minimal white flecking causes the throat and chest to appear uniform gray or
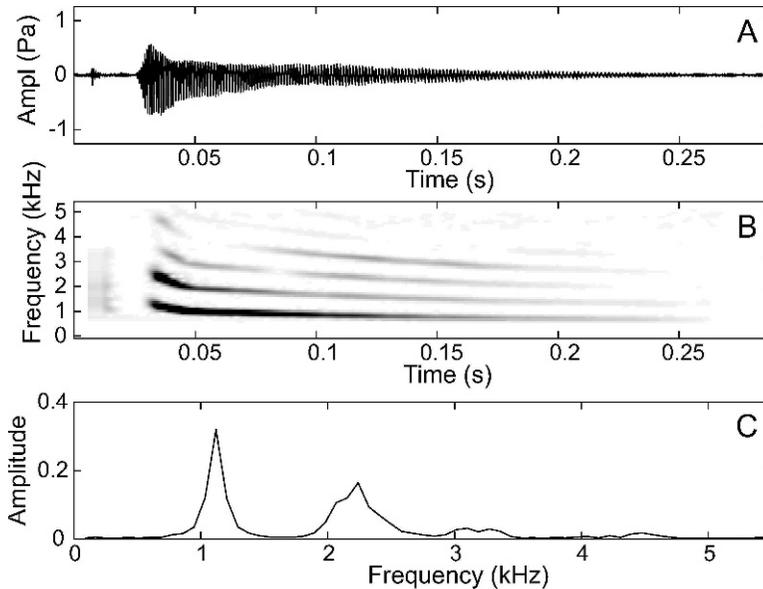
Fig. 2.—(A) Oscillogram, (B) spectrogram, and (C) power spectrum of the advertisement call of *Physalaemus petersi* (QCAZ 28581). The power spectrum was measured along the entire length of the call.

brown (QCAZ 26215). A cream midventral stripe extends from the tip of the snout to the belly, although this is faint in some individuals (KU 222070).

Mean SVL was significantly larger for females than males ($t = 8.17$, df $= 95$, $P <$ 0.001; Table 1). Significant variation was found in mean SVL among sites in both males ($F = 51.01$, df $= 122$, $P < 0.001$) and females ($F = 8.48$, df $= 51$, $P < 0.001$). Mean male SVL ranged from 22.8 mm at Shell, Provincia Napo, Ecuador ($n = 3$), to 30.9 mm at Puerto

TABLE 2.—Call parameters of *Physalaemus petersi* and *P. freibergi*. Abbreviations are $n =$ number of males; ALLDUR $=$ duration of entire call; ALLDOMHZ $=$ dominant frequency of entire call; WDUR $=$ duration of whine; WDOMHZ $=$ dominant frequency of whine; MAXHZ $=$ maximum frequency of whine; INITHZ $=$ initial frequency of whine; MSHFHZ $=$ time to half maximum frequency of whine; FINHZ $=$ final frequency of whine; RISET $=$ time to maximum amplitude; HALFRISET $=$ time to half maximum amplitude; FALLT $=$ time from maximum amplitude to end of call; HALFFALLT $=$ time from maximum amplitude to half maximum amplitude; PDUR $=$ duration of prefix; PDOMHZ $=$ dominant frequency of prefix. All frequencies are in Hz and durations in ms.

| Parameter	*P. petersi*		*P. freibergi*	
	Mean ± SD	Min–max	Mean ± SD	Min–max
n	73		31	
ALLDUR	253.59 ± 63.78	43.00–378.32	116.10 ± 29.72	49.86–166.28
ALLDOMHZ	590.5 ± 179.0	396.7–1550.4	478.4 ± 155.2	299.1–1012.1
WDUR	213.91 ± 68.07	32.31–346.15	96.72 ± 28.83	36.49–148.48
WDOMHZ	569.1 ± 186.4	293.0–1464.3	446.3 ± 52.5	371.5–567.6
MAXHZ	975.2 ± 173.2	535.7–1378.0	844.4 ± 99.7	549.5–1025.3
INITHZ	950.3 ± 153.1	535.7–1378.0	844.4 ± 99.7	549.5–1025.3
MSHFHZ	19.61 ± 20.00	4.99–167.12	7.70 ± 4.37	1.19–18.56
FINHZ	442.9 ± 117.6	258.7–688.6	318.6 ± 81.3	97.9–585.9
RISET	8.69 ± 8.49	2.83–45.31	5.15 ± 1.90	2.72–11.08
HALFRISET	2.74 ± 2.51	0.00–18.00	1.98 ± 0.86	1.00–4.92
FALLT	229.27 ± 60.84	25.67–360.20	91.64 ± 28.55	32.34–144.76
HALFFALLT	30.54 ± 29.71	10.43–137.73	19.88 ± 15.81	10.08–82.12
PDUR	9.56 ± 7.79	0.60–62.09	6.70 ± 2.68	3.29–15.48
PDOMHZ	915.6 ± 549.9	129.2–4687.5	639.8 ± 226.4	172.3–1033.6
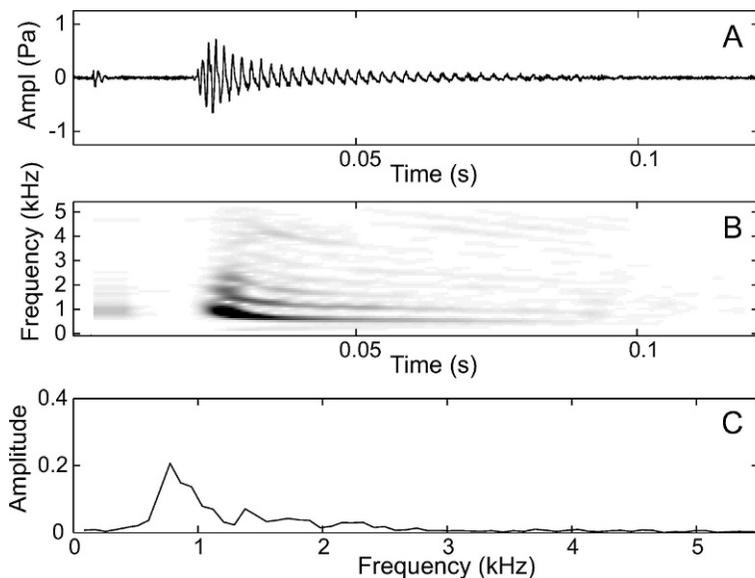
F_IG. 3.—(A) Oscillogram, (B) spectrogram, and (C) power spectrum of the advertisement call of *Physalaemus freibergi* (MUSM 19402). The power spectrum was measured along the entire length of the call.

Bolívar, Provinicia Sucumbíos, Ecuador (*n* = 2). Mean female SVL ranged from 27.9 at San Jacinto, Departamento Loreto, Peru (*n* = 6), to 36.6 at Huampami, Departamento Amazonas, Peru (*n* = 2). All morphometric variables (Table 1) were significantly correlated with SVL at the *P* < 0.001 level for both males (r^2 = 22.1% for eye–nostril distance to 77.1% for tibia length) and females (r^2 = 22.0% for eye–nostril distance to 78.8% for tibia length).

Distribution and ecology.—Physalaemus petersi occurs in Amazonian Ecuador, northeastern Peru, and Amazonian Colombia from 89 to 1069 m based on our analysis of mtDNA, museum specimens, and advertisement calls (Fig. 5). All sites north of Río Amazonas and Río Marañon, except for two sites in northern Peru (Explorama Lodge in Departamento Loreto and Galilea in Departamento Amazonas), were assigned to *P. petersi* based on morphology using Discrim-

F_IG. 4.—Dorsal views of adult *Physalaemus petersi*, showing variation in dorsal patterns. Left to right: QCAZ 15118 (male from Estación Científica de la Universidad Católica del Ecuador, Parque Nacional Yasuní, Provincia Orellana), QCAZ 26215 (male from Puyo, Provincia Napo), and QCAZ 28596 (female from La Selva Hostería, Provincia Sucumbíos). All are from Ecuador.
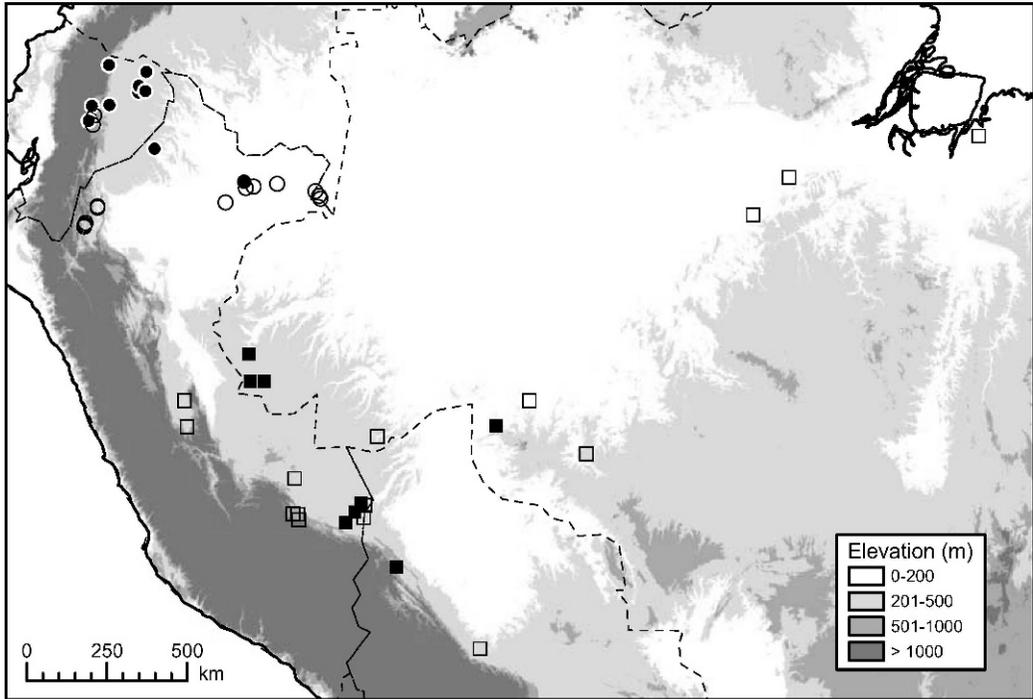
Fig. 5.—Known records of *Physalaemus petersi* (circles) and *P. freibergi* (squares). Solid symbols are sites with known haplotypes (based on Funk et al., 2007) and open symbols are sites assigned to *P. petersi* or *P. freibergi* based on morphology and/or calls using Discriminant Function Analysis.

inant Function Analysis. However, assignment of Explorama to *P. freibergi* was based on only one specimen, and assignment of Galilea to *P. freibergi* (mean posterior probability of 0.55) over *P. petersi* (mean posterior probability of 0.45) was poorly supported. Moreover, the 12 sites surrounding Explorama and Galilea were assigned to *P. petersi*. Thus, the data suggest that the border between *P. petersi* and *P. freibergi* lies at or south of the Río Amazonas and Río Marañon. Molecular, morphological, and call analysis of "*P. petersi*" from French Guiana (Cannatella and Duellman, 1984; Lescure and Marty, 2000) is needed to determine whether these frogs are *P. petersi*, *P. freibergi*, or an undescribed species.

Physalaemus petersi is primarily an inhabitant of primary humid rainforest and Andean foothills, although it occasionally uses secondary forest, forest edge, and clearings (Duellman, 1978). Throughout its known range, *P. petersi* does not occur in sympatry with any other *Physalaemus* species. Reproductive activity is nocturnal and choruses are found along the edges of oxbow lakes, ponds, and pools (Duellman, 1978; Rodríguez and Duellman, 1994). Amplexus and egg deposition occur at the same sites where males call. As in other *Physalaemus*, *P. petersi* constructs foam nests that are formed during amplexus as the male beats the eggs with his legs (Rodríguez and Duellman, 1994). The diet of *P. petersi* consists entirely of termites (Duellman, 1978). *Physalaemus petersi* appears to be distributed patchily throughout lowland rainforest. It can be abundant at some sites (Duellman and Mendelson, 1995), but is absent from other seemingly appropriate sites.

Call.—The "simple" call of *Physalaemus petersi* consists of two components: a short, low-amplitude "prefix" followed by a longer, frequency-modulated "whine" with four or more harmonics (Fig. 2; Table 2). The prefix has a mean duration of 9.56 ms (range 0.60–62.09 ms) and a mean dominant frequency of 915.6 Hz (range 129.2–4687.5 Hz). The whine has a mean duration of 213.91 ms (range 32.31–346.15 ms) and dominant fre-

quency of 569.1 Hz (293.0–1464.3 Hz). The whine has a downward frequency sweep, starting at a mean frequency of 950.3 Hz (535.7–1378.0 Hz) and ending at a mean frequency of 442.9 Hz (258.7–688.6 Hz). Males from two populations of *P. petersi*, Estación Científica Yasuní and Tiputini Biodiversity Station, can facultatively add a third call component, the "squawk", to produce a "complex" call (Boul et al., 2007). The "squawk" of *P. petersi* is analogous, and perhaps homologous, to the "chuck" of *P. pustulosus* (Ryan, 1985). In both the squawk and chuck, the fundamental frequency is usually half the fundamental frequency of the whine and the upper harmonics have more energy than the upper harmonics of the whine.

Remarks.—*Physalaemus petersi* is assigned to the clade Edentulus sensu Ron et al. (2006) based on three morphological synapomorphies: (1) first finger equal in length or longer than second; (2) teeth on the maxilla and premaxilla absent; and (3) anterior process of hyale weakly developed (Cannatella et al., 1998). Although Nascimento et al. (2005) resurrected the genus *Engystomops* for the *Physalaemus pustulosus* group, that action is not consistent with their own analysis of relationships. Ron et al. (2006) followed the use of *Engystomops*. However, one of the authors of the latter and the current papers (DCC) agrees that the resurrection of *Engystomops* as a genus was unjustified and has a larger manuscript in preparation on the molecular systematics of *Physalaemus*. Therefore the use of *Physalaemus* is continued here.

Physalaemus freibergi (*Donoso-Barros*)

Eupemphix freibergi Donoso-Barros, 1969; holotype Donoso-Barros collection 745, now at the Universidad de Concepción, Instituto de Zoología, Concepción, Chile, from "Runerrabaque, Río Beni, Bolivia" (= Rurrenabaque, Departamento El Beni, Bolivia, according to Cannatella and Duellman, 1984).

Physalaemus freibergi Cannatella, Hillis, Chippindale, Weigt, Rand, and Ryan, 1998 *Diagnosis.*—A member of the genus *Physalaemus* and the *P. pustulosus* group, sensu Cannatella and Duellman (1984) and Cannatella et al. (1998); see Remarks. Assignment to the *P. pustulosus* group is based on the presence of four synapomorphies (Cannatella et al., 1998): (1) presence of flank glands; (2) presence of paratoid glands; (3) warty skin; and (4) dentigerous process of the vomer thin and spikelike.

Physalaemus freibergi (Fig. 6) is characterized by: (1) mean SVL 27.8 mm in males (range 23.5–35.7; n = 79), 31.6 mm in females (range 25.0–39.1; n = 45); (2) skin on dorsum bearing small tubercles with scattered larger tubercles, sometimes forming diverging rows in the occipital and scapular regions; (3) snout protruding with anterior margin of lip at a position posterior to the nostrils; (4) vomerine teeth and odontophores absent; (5) maxillary teeth absent; (6) paratoid glands present; (7) flank glands present; (8) tarsal tubercle present; (9) nuptial pads present; (10) Finger I longer than II; (11) tympanic annulus usually well-defined at least ventrally, tympanum smooth; (12) dentigerous process of the vomer thin and spikelike.

The presence of a tarsal tubercle and absence of maxillary teeth distinguishes *P. freibergi* from all trans-Andean species in the *Physalaemus pustulosus* species group (clade name Duovox sensu Ron et al., 2006), which includes *P. pustulatus*, *P. guayaco*, *P. montubio*, *P. coloradorum*, and *P. randi*. These species in the lowlands of western Ecuador and northwestern Peru also have Finger I shorter than or equal in length to II (Finger I longer than II in *P. freibergi*). *Physalaemus pustulosus* differs from from *P. freibergi* by having a more elongate gland on the flank, a snout with the anterior margin of the lip at the level of the posterior margin of the nostrils, a tuberculate tympanic membrane, and a pattern of tubercles on the dorsum consisting of randomly scattered large and small tubercles or the large tubercles forming longitudinal rows or chevrons. *Physalaemus pustulosus* also lacks the large black inguinal spots characteristic of *P. freibergi* and *P. petersi*. *P. freibergi* differs from *P. petersi* in advertisement calls and morphology. Mean call duration is significantly shorter in *P. freibergi* (mean = 116.1 ms, SD = 29.7 ms, n = 31; Fig. 3; Table 2) than *P. petersi* (mean = 253.6 ms, SD = 63.8 ms, n = 73, t = 14.98, df = 101, P < 0.001; Fig. 2). Mean call
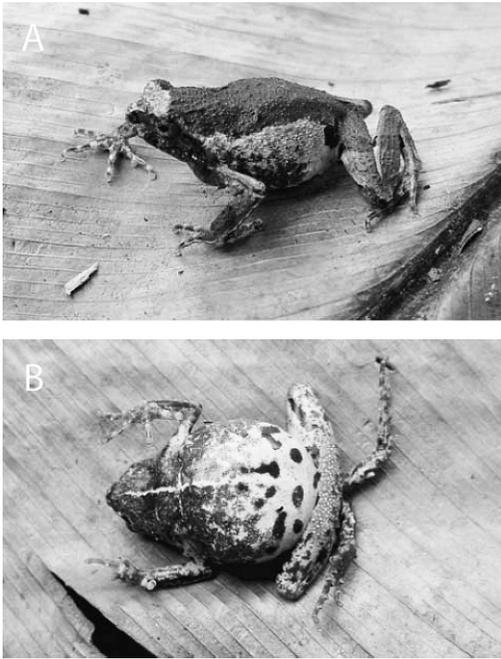
Fig. 6.—Dorsolateral (A) and ventral (B) views of an adult male *Physalaemus freibergi*, MUSM 19368 (from Tambopata Research Center, Departamento de Madre de Dios, Peru).

frequency is significantly lower in *P. freibergi* (mean = 478 Hz, SD = 155 Hz, *n* = 31) than *P. petersi* (mean = 591 Hz, SD = 179 Hz, *n* = 73, *t* = 3.21, df = 64, *P* = 0.002). *Physalaemus freibergi* also has proportionately shorter legs and a wider dorsum and head than *P. petersi* (see Morphometric comparisons below).

Variation.—Dorsal coloration is variable in preserved specimens of *P. freibergi* (Fig. 7). Most of the variation is in: (1) number, distribution, and size of tubercles; (2) presence or absence of tubercles forming rows in the occipital and scapular regions; (3) hue of the background coloration, varying from tan to dark grey or dark brown; (4) presence of chevrons or dark interorbital bars; and (5) presence or absence of pale snouts and flanks, the "pale morph" as defined by Cannatella and Duellman (1984). In *P. freibergi*, the frequency of the pale morph was not significantly different between females (21.6%, *n* = 45) and males (19.7%, *n* = 79, Fisher's exact test, *P* = 1.00).

Ventral surfaces of all preserved specimens have a cream or white background with dark markings on the abdomen and gray chests and throats suffused with white flecks (Fig. 6). The color of the markings on the abdomen is generally dark gray to black. The size and number of these dark marks increases toward the groin. The amount of white flecking increases toward the posterior of the chest where it blends with the cream abdomen. A cream midventral stripe extends from the tip of the snout to the belly.

Mean SVL was significantly larger for females than males (*t* = 5.73, df = 74, *P* < 0.001; Table 1). Significant variation was found in mean SVL among sites in both males (*F* = 24.27, df = 74, *P* < 0.001) and females (*F* = 3.96, df = 41, *P* = 0.002). Mean male SVL ranged from 24.8 mm at Tambopata Research Centre, Departamento de Madre de Dios, Peru (*n* = 12), to 35.4 mm at Agropecuária Treviso, state of Pará, Brazil (*n* = 3). Mean female SVL ranged from 28.7 at Tambopata Research Centre (*n* = 8), to 36.4 at Rio Formoso, Parque Estadual Guajará-Mirim, state of Rondônia, Brazil (*n* = 4).

Distribution and ecology.—*Physalaemus freibergi* occurs in Amazonian Brazil, northern Bolivia, and southeastern Peru from 10 to 999 m (Fig. 5). All sites south of the Río Amazonas and Río Marañon, except for one site in central Peru (Nevati in Departamento de Pasco), were assigned to *P. freibergi* based on morphology and/or calls using Discriminant Function Analysis. Assignment of Nevati to *P. petersi* was based on only one specimen, and all surrounding sites were assigned to *P. freibergi*. The data therefore suggest that the boundary between *P. petersi* and *P. freibergi* lies somewhere between the Rio Amazonas and the state of Acre in western Brazil.

Like *P. petersi*, *P. freibergi* is primarily an inhabitant of primary humid rainforest (Aichinger, 1992; Parmelee, 1999; Rodríguez and Cadle, 1990; Toft and Duellman, 1979). *Physalaemus freibergi* occurs in sympatry with *P. ephippifer* in the central and eastern Amazon (Caldwell and Araújo, 2005). Reproductive activity is nocturnal and breeding occurs in ponds, swamps, forest pools, lakes, and slow-moving streams (Aichinger, 1987; Rodríguez and Cadle, 1990). *Physalaemus freibergi* also constructs foam nests (Parmelee, 1999; Rodríguez and Cadle, 1990; Toft and

FIG. 7.—Dorsal views of adult *Physalaemus freibergi*, showing variation in dorsal patterns. Left to right: USNM 303914 (male from Alto Paraíso), OMNH 37268 (male from Rio Formoso, Parque Estadual Guajará-Mirim), OMNH 37281 (male from Rio Formoso, Parque Estadual Guajará-Mirim), and OMNH 37275 (female from Rio Formoso, Parque Estadual Guajará-Mirim). All are from Estado de Rondônia, Brazil.

Duellman, 1979). The diet of *P. freibergi* is 99% termites, both in terms of volume and number (Parmalee, 1999). We dissected the stomachs of two *P. freibergi* found foraging at termite columns at Tambopata Research Centre, Departamento de Madre de Dios, Peru, and found 46 (MUSM 19368) and 89 (MUSM 19369) termites. *Physalaemus freibergi* is rare to common across its range (Aichinger, 1992; Doan and Arizábal, 2002; Duellman, 1995; Duellman and Salas, 1991; Parmalee, 1999; Toft and Duellman, 1979).

Call.—As with *P. petersi*, the simple call of *Physalaemus freibergi* has a prefix and a whine component (Fig. 3; Table 2). The prefix has a mean duration of 6.70 ms (range 3.29–15.48 ms) and a mean dominant frequency of 639.8 Hz (range 172.3–1033.6 Hz). The whine has a mean duration of 96.72 ms (range 36.49–148.48 ms) and dominant frequency of 446.3 Hz (371.5–567.6 Hz). The whine has a downward frequency sweep, starting at a mean frequency of 844.4 Hz (549.5–1025.3 Hz) and ending at a mean frequency of 318.6 Hz (97.9–585.9 Hz). Males from three populations, Boca do Rio Tejo and Restauraçao (both in the state of Acre, Brazil) and Explorer's Inn (in Departamento de Madre de Dios, Peru), have complex calls with a third squawk component as described above for *P. petersi* (Boul et al., 2007).

Remarks.—*Physalaemus freibergi* is also assigned to the clade Edentulus sensu Ron et al. (2006) based on the three morphological synapomorphies described above.

Morphometric comparisons between P. petersi *and* P. freibergi.—Measurements of 10 morphometric variables are shown in Table 1. In the PCA for males, the first three principal components account for 59.7% of the variation (Table 3). Principal component (PC) I explains a gradient based primarily on tarsus and tibia length, whereas PC II explains a gradient based on head and dorsum width. PC I scores were significantly larger in *P. petersi* than *P. freibergi* ($t = 9.03$, df = 147, $P < 0.001$), indicating that the tarsus and tibia were proportionately longer in *P. petersi* than *P. freibergi* (Fig. 8). PC II scores were also significantly larger in *P. petersi* than *P. freibergi* ($t = 3.99$, df = 167, $P < 0.001$), indicating that the dorsum and head were proportionately narrower in *P. petersi*. In the PCA for females, the first three principal components accounted for 64.6% of the variation. PC I had a high loading for tibia length, and PC II had high positive loadings for dorsum and head width and high negative loadings for tarsus and tubercle–heel length. PC I scores were significantly larger in *P. petersi* than *P. freibergi* ($t = 2.66$, df = 95, $P = 0.009$), indicating that the tibia was proportionately longer in *P. petersi* than *P. freibergi*. PC II scores were significantly smaller in *P. petersi* than *P. freibergi* ($t = 5.19$, df = 102, $P < 0.001$), indicating that the dorsum and head were proportionately nar-

TABLE 3.—Character loading and percentage of the variance explained by Principal Components (PC) I–III for 9 morphometric variables. To remove the effects of "size", linear regressions were performed between all variables and snout–vent length. The PC analysis was applied to the residuals from these regressions.

Variable	Males ($n = 206$)			Females ($n = 105$)		
	PC I	PC II	PC III	PC I	PC II	PC III
Resid dorsum width	−0.164	**−0.496**	0.136	0.319	**0.434**	−0.084
Resid tibia length	**0.507**	−0.153	0.061	**0.469**	−0.238	0.153
Resid femur length	0.017	−0.399	**−0.509**	0.396	0.098	0.021
Resid arm length	0.329	−0.193	−0.376	0.397	−0.075	0.121
Resid head length	0.035	−0.454	**0.513**	0.258	0.191	**−0.732**
Resid head width	−0.220	**−0.560**	0.025	0.270	**0.412**	−0.094
Resid eye-nostril distance	−0.158	−0.093	**−0.553**	0.257	0.228	0.563
Resid tarsus length	**0.560**	−0.065	0.079	0.363	**−0.472**	0.050
Resid tubercle-heel length	0.470	−0.040	−0.042	0.164	**−0.508**	−0.302
Eigenvalue	2.58	1.66	1.13	3.02	1.87	0.93
%	28.7	18.4	12.6	33.6	20.7	10.3

rower and the tarsus was proportionately longer in *P. petersi*. Thus, although overlap was substantial between *P. petersi* and *P. freibergi* in morphometric space, significant

FIG. 8.—Axes I and II from Principal Components Analysis based on nine size-corrected morphological variables for (A) male *Physalaemus petersi* ($n = 127$) and *P. freibergi* ($n = 79$), and (B) female *P. petersi* ($n = 60$) and *P. freibergi* ($n = 45$). Open circles and dotted lines = *P. petersi*; filled circles and dashed lines = *P. freibergi*.

differences were found in leg length and head and dorsum width.

DFA of morphometric measurements using all specimens assigned most individuals to the correct species (Table 4). For males, 87.4% of *P. petersi* were correctly assigned and 82.3% of *P. freibergi* were correctly assigned. For females, 76.7% and 80.0% of *P. petersi* and *P. freibergi* were correctly classified, respectively. These results show that both species can be correctly assigned to species most of the time based on morphometric variables alone.

Call comparisons between P. petersi *and* P. freibergi.—Measurements of 14 advertisement call variables are shown in Table 2. In the PCA, the first three principal components account for 66.4% of the variation (Table 5). Principal component (PC) I explains a gradient based primarily on frequency, whereas PC II explains a gradient based on call duration. PC I scores were significantly larger in *P. petersi* than *P. freibergi* ($t = 10.65$, df = 101, $P < 0.001$), indicating higher frequency calls in *P. petersi* than *P. freibergi* (Fig. 9). PC II scores were also significantly larger in *P. petersi* than *P. freibergi* ($t = 6.23$, df = 100, $P < 0.001$), indicating that the calls of *P. petersi* were longer. DFA of acoustic parameters using all recorded males assigned almost all individuals to the correct species (Table 6). 100.0% of *P. petersi* were correctly assigned and 96.8% of *P. freibergi* were correctly assigned. Thus, species can be classified more accurately using calls than morphology.
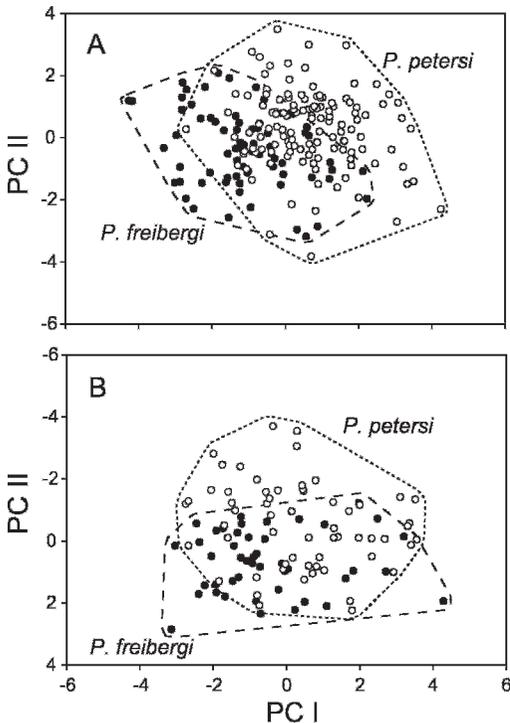
TABLE 4.—Summary of the number of individuals correctly classified as *Physalaemus petersi* or *P. freibergi* using Discriminant Function Analysis of all 10 morphometric variables.

| | Males | | Females | |
| | True group | | True group | |
Assigned to	*P. petersi*	*P. freibergi*	*P. petersi*	*P. freibergi*
P. petersi	111	14	46	9
P. freibergi	16	65	14	36
Total number	127	79	60	45
Number correct	111	65	46	36
%	87.4	82.3	76.7	80.0

DISCUSSION

Molecular, morphological, and call data all support recognition of *P. petersi* and *P. freibergi* as separate species. *Physalaemus petersi* has proportionately longer legs and a narrower dorsum and head than *P. freibergi*, and the calls of *P. petersi* are higher in frequency and longer than *P. freibergi*. It is also possible that there are cryptic species within *P. petersi* or *P. freibergi*, but more data are required to test this. In particular, *P. petersi* populations on opposite sides of the Río Napo in eastern Ecuador (La Selva, Provincia Sucumbíos, on the North side and Estacíon Cientíica Yasuní and Tiputini Biodiversity Station, Provincia Orellana, on the South) have diverged in calls, female preferences for calls, and morphology, and thus represent incipient species (Boul et al., 2007). At this point, however, the distribution of these incipient species is uncertain. Also, at two sites (San Jacinto, Departamento de Loreto, Peru, and Puerto Bolívar, Provincia Sucumbíos, Ecuador), divergent haplotypes were uncovered which may represent different species living sympatrically (Funk et al., 2007). Recent studies suggest that it may not be uncommon for related species of Neotropical frogs to occur sympatrically that are only revealed by call and molecular differences (Angulo et al., 2003; Ron et al., 2004, 2005).

Phylogeographic analysis also revealed a third divergent haplotype in the central Amazon Basin (the "Pará" clade) that may represent a third species (Funk et al., 2007). However, in the current analysis, Discriminant Function Analysis grouped individuals from this site and other sites in the central and eastern Amazon Basin with *P. freibergi*. Additional sequence, morphological, and call data from Amazonian Brazil and French Guiana will be necessary to determine whether there are additional species in the *Physalaemus pustulosus* group in the central and eastern Amazon Basin.

TABLE 5.—Character loading and percentage of the variance explained by Principal Components (PC) I–III for 14 call variables. Call abbreviations are the same as in Table 2. Analysis based on calls from 104 males.

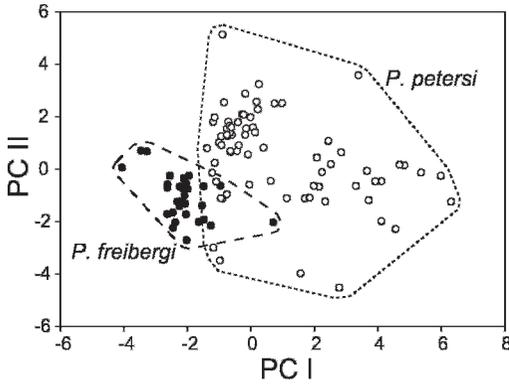| Variable	PC I	PC II	PC III
ALLDUR	0.253	**0.468**	−0.004
ALLDOMHZ	0.265	−0.286	**0.397**
WDUR	0.290	**0.383**	0.044
WDOMHZ	0.260	−0.217	0.344
MAXHZ	**0.389**	−0.203	−0.055
INITHZ	**0.369**	−0.165	−0.126
MSHFHZ	0.116	0.238	0.326
FINHZ	**0.343**	−0.222	0.195
RISET	0.311	−0.064	−0.346
HALFRISET	0.186	0.057	−0.389
FALLT	0.226	**0.478**	0.049
HALFFALLT	0.313	−0.132	−0.257
PDUR	0.115	0.210	−0.152
PDOMHZ	0.052	0.183	**0.443**
Eigenvalue	5.01	2.80	1.48
%	35.8	20.0	10.6

Fig. 9.—Axes I and II from Principal Components Analysis based on 14 call variables for *Physalaemus petersi* (*n* = 73 calls from different males) and *P. freibergi* (*n* = 31). Open circles and dotted lines = *P. petersi*; filled circles and dashed lines = *P. freibergi*.

Resumen

Analizamos y describimos diferencias cuantitativas en la morfología y los cantos de anuncio de *Physalaemus petersi* y *P. freibergi*, ambos miembros del grupo monofilético de especies *Physalaemus pustulosus*. Encontramos diferencias significativas entre las dos especies en parámetros morfométricos y de cantos de anuncio. *Physalaemus petersi* tiene piernas proporcionalmente más largas y un dorso y cabeza más estrechos que *P. freibergi*. Los cantos de *P. petersi* son más altos en frecuencia y más largos que los de *P. freibergi*. Un Análisis de Función Discriminante (AFD) de variables morfométricas clasificó correctamente 76.7–87.4% de los individuos a cada especie. La AFD de variables de cantos clasificó correctamente 96.8–100.0% de machos a cada especie. *Physalaemus petersi* se distribuye al norte del Río Marañon y del Río Amazonas en el este de Ecuador, noreste de Perú, y sureste de Colombia; *P. freibergi* se distribuye al sur de estos ríos en la Amazonía de Brasil, el sureste de Perú, y la Amazonía de Bolivia. Los cantos y la ubicación geográfica son la manera más confiable de identificar estas especies en el campo.

TABLE 6.—Summary of the number of individuals correctly classified as *Physalaemus petersi* or *P. freibergi* using Discriminant Function Analysis of all 14 call variables.

	True group	
Assigned to	*P. petersi*	*P. freibergi*
P. petersi	73	1
P. freibergi	0	30
Total number	73	31
Number correct	73	30
%	100.0	96.8

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APPENDIX I

Examined Specimens

Physalaemus freibergi.—BOLIVIA: DEPARTAMENTO DE COCHABAMBA: 6.5 km N Chipiri, 260 m (KU 135513–14). BRAZIL: ESTADO DO ACRE: 5–10 km N Porto Walter, inland from Rio Juruá, 219 m (OMNH 36487–88, 36493–95, 36498–500). ESTADO DO PARÁ: Agropecuária Treviso, 122 m (OMNH 34811–13); Belém, 10 m (KU 127320–26); Itaituba, 66–75 km SW Parque Nacional da Amazônia, Rio Tapajós, 44 m (USNM 241210–11). ESTADO DO RONDÔNIA: Alto Paraíso, 126 m (USNM 303911–14); Nova Brasilia, 433 m (USNM 304115); Rio Formoso, Parque Estadual Guajará-Mirim, 90 km N Nova Mamoré, 151 m (OMNH 37268–72, 37274–81). PERÚ: DEPARTAMENTO DE CUSCO: Pilcopata, 999 m (KU 138979). DEPARTAMENTO DE HUÁNUCO: Finca Panguana, Río Llullapichis, 4–5 km upstream from Río Pachitea, 260 m (KU 154902, 171903). DEPARTAMENTO DE MADRE DE DIOS: Hacienda Amazonía on west bank of Río Alto Madre de Dios, 3 km NW Atalaya, 500 m (USNM 346159–61, 346390); Cusco Amazónico, 15 km E Puerto Maldonado, 200 m (KU 215534); Explorer's Inn, Tambopata National Reserve, 30 km SSW Puerto Maldonado, 207 m (USNM 222305–08, 247425, 247428–30, 247659, 268989, 268993, 268995–97, 343004–05, 343007–08, 343260–65); Hacienda Erika, NW bank Río Alto Madre de Dios at Salvación, 671 m (MVZ 196917, 196919); Pakitza on Río Manu, Reserve Zone, Manu National Park, 57 km NW mouth of Río Manu, 350 m (USNM 342648–49, 342861, 345291); Sanctuary of Las Pampas del Río Heath, 160 m (USNM 314903–04); Río Palma Real Grande, Santuario Nacional Pampas del Heath, Puerto Enahuipa, 216 m (MCZ 136358, 136362); Lago Sandoval to trail between Río Madre de Dios and Lago Sandoval, 200 m (KU 215133, MVZ 173730); Tambopata Research Centre on north side of Río Tambopata, 167 m (MUSM 19338, 19341–43, 19346, 19349–54, 19357, 19362, 19366, 19370–73, 19377, 19404); Tambopata Research Centre on island on south side of Río Tambopata, 201 m (MUSM 19339, 19347–48, 19375, 19380, 19382–83, 19385–86, 19390–93, 19395–96, 19398, 19400–02). DEPARTAMENTO DE PASCO: Nevati, 275 m (KU 144317).

Physalaemus petersi.—COLOMBIA: DEPARTAMENTO DE AMAZONAS: 50 km NW Puerto Nariño, 117 m (MCZ 94962); Puerto Nariño, 89 m (MCZ 94963); Río Amacayacu, tributary of Río Amazonas, 95 m (MCZ 96861–62). ECUADOR: PROVINCIA DE ORELLANA: Estación Científica Yasuní de la Pontificia Universidad Católica del Ecuador, 250 m (QCAZ 15109, 15112, 15118, 15120, 15123, 15125, 15127–31, 15134, 15139–40, 20281); Tiputini Biodiversity Station of the Universidad San Francisco de Quito, 208 m (QCAZ 28629). PROVINCIA DE PASTAZA: Finca de Medardo Tapia, 6 km towards Puyo from the Y (km 31) Vía Puyo-Tena, 973 m (QCAZ 25329); Fundación Hola Vida, 846 m (QCAZ 25612, 25725); Hostería Safari, 5 km N Puyo on road to Tena, 954 m (QCAZ 26210–12, 26215–16, 26218, 26221–24, 26226–29, 26247, 26250, 26253–54, 26258, 26263, 26265, 26277, 26294); Shell, Iwia training camp, 1069 m (QCAZ 25038–25040). PROVINCIA DE SUCUMBÍOS: Hostería La Selva, 226 m (QCAZ 28576–86, 28588–89, 28591–99, 28601–02, 28604–06); Comunidad Asociación Chonta Yacu, parroquia Lumbaqui, 610 m (QCAZ 25790); Puerto Bolívar, Cuyabeno, 240 m (QCAZ 28139, 28148, 28167, 28169, 28172, 28187, 28200, 28204, 28208, 28255). PERÚ: DEPARTAMENTO DE AMAZONAS: Galilea, Río Santiago, 180 m (MVZ 173926–35, 173992–4001); vicinity of Huampami, Río Cenepa, 270 m (MVZ 162692–711); vicinity of San Antonio, Río Cenepa, 480 m (MVZ 162737); Shiringa, Quebrada Yutipis, Río Santiago, 223 m (MVZ 173936–39, 173941–55); vicinity of Sua, Río Cenepa, 341 m (MVZ 162734–36, 162739). DEPARTAMENTO DE LORETO: Amazon Conservatory for Tropical Studies (ACTS), 102 m (MUSM 21543, 21546, 21549–51, 21557–58, 21568–70, 21572); Explorama Lodge, confluence of Río Yanamono and Río Amazonas, 98 m (KU 220477); Mishana, Río Nanay, 100 m (MCZ 88915–16, TCWC 53375); Quebrada Orán, 5 km N Río Amazonas, 85 km NE Iquitos, 110 m (KU 206122–25); Pevas [Pebas], Río Ampiyacu, 97 m (CAS 6316, 6319, MCZ 25598); San Jacinto, 180 m (KU 222059–64, 222068–70, 222072–74, 222076–77).

Physalaemus pustulatus.—ECUADOR: PROVINCIA DE MANABÍ: Puerto Rico, 10 m (QCAZ 19513–14, 19518).

Physalaemus pustulosus.—PANAMÁ: COLÓN: Gamboa (TNHC 62667–69).

Physalaemus randi.—ECUADOR: PROVINCIA DEL GUAYAS: 11 km N Cerro Masvale, 40 m (QCAZ 23461, 23523); Cerro Masvale, 92 m (19558–560).