



A new cryptic species of the genus *Eleutherodactylus* (Amphibia: Anura: Eleutherodactylidae) from Cuba

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Abstract

The widely distributed grass frog of Cuba, *Eleutherodactylus varleyi*, is shown here to comprise two species. One, *E. varleyi*, occurs in western and central Cuba while the other, *E. feichtingeri* n. sp., occurs in central and eastern Cuba. The two species are sympatric in central Cuba, and syntopic in the vicinity of Sierra de Cubitas, Camagüey Province. A molecular phylogeny of mitochondrial DNA sequences from 18 localities confirms the existence of two well-supported major clades corresponding to each of these species, and the sympatry of the species. Tympanum size and advertisement call are the most useful diagnostic characters, although the two species are shown to have karyotypic differences as well. Possible character displacement in morphology and vocalization, in the area of sympatry, is discussed.

Key words: Caribbean, West Indies, Terrarana, *Euhyas*, morphology, phylogeny, bioacoustics, chromosomes

Introduction

Cuban frogs are represented by 65 species, following the general consensus of different authors (Henderson and Powell, 2009; Schmid *et al.*, 2010; Frost, 2010). The genus *Eleutherodactylus* comprises 85% of the Cuban anuran fauna. Within the genus, Hedges *et al.* (2008) defined species series and species groups in their revision of the very large Neotropical frog clade Terrarana. Group placement and phylogenetic relationships among species were supported by DNA sequence data, data in some cases were inferred from shared morphological characters when molecular information was not available. In the Cuban *Eleutherodactylus gundlachi* species group, of the *Eleutherodactylus (Euhyas) planirostris* species series, these authors included the Cuban species *E. adelus* Díaz, Cádiz, and Hedges, 2003; *E. gundlachi* Schmidt, 1920; *E. intermedius* Barbour and Shreve, 1937; *E. tetajulia* Estrada and Hedges, 1996; and *E. varleyi* Dunn, 1925.

Eleutherodactylus varleyi is one of the widely distributed frogs in Cuba, inhabiting both open lowlands and mountain forests, from sea level to approximately 900 m altitude (Schwartz and Henderson, 1991; Díaz and Cádiz, 2008). Díaz *et al.* (2003), and Díaz and Cádiz (2007, 2008) recognized two different advertisement call patterns in *E. varleyi*. Pattern I was considered as typical for the species and basically consists of sequences of two-note calls. Pattern II was characterized by single-note calls with a higher intensity than those present in Pattern I. These authors suggested that call patterns might be related to the presence of at least two cryptic species. Additionally, Schmid *et al.* (2010) found karyotypic differences between western (acoustic Pattern I) and eastern (Pattern II) populations of *E. varleyi*, which support this hypothesis. During September–October of 2002 and 2010, both call patterns were found at the surroundings of Sierra de Cubitas. Sympatric specimens with either kind of advertisement call also had morphological differences. A review of several populations of *E. varleyi* throughout Cuba, combining morphology, bioacoustics, DNA sequences, and cytogenetic analyses, provides enough evidence for the recognition of a new species, which is herein described and named.

Material and methods

Measurements were taken with calipers (0.05 mm accuracy) and an ocular micrometer (0.1 mm) under a Wild-M5 dissecting microscope. Head length was measured from the snout tip to the mandibular articulation. Interorbital distance was taken at a middle point between inner margins of the eyelids. Snout length comprises the distance from the snout tip to the anterior orbital border.

Calls were recorded with a Marantz PMD 222 professional cassette recorder, a Sennheiser ME 66 microphone, and Type II tapes (60 minutes). Acoustic analysis was performed with the software BatSound 2.1 (Pettersson Elektronik AB, © 1996–1999). Signals were digitized at 44.1 kHz and sample size of 16 bits. Sonograms were generated with a FFT (Fast Fourier Transform) of 512 points, using Hanning windows. Call rise time (and note rise time) were measured from the beginning of the signal to the point of maximal amplitude. Temporal variables were all measured in the oscillograms, and the dominant frequency was taken as the peak frequency in the power spectrum (as shown in Díaz and Cádiz, 2007).

An 892-base pair portion of the cytochrome b (Cyt-b) mitochondrial gene was sequenced in 48 individuals of *E. varleyi* (*sensu lato*), representing 18 localities across Cuba. DNA extraction, sequencing, and phylogenetic analysis was accomplished using standard methodology, as described elsewhere (Heinicke *et al.*, 2007; Hedges *et al.*, 2008). A tree was constructed with MEGA 5.0 (Tamura *et al.*, 2011) using maximum likelihood (general time reversible + gamma + invariants model) and 2000 bootstrap replications. The following are localities and sequence accession numbers (GenBank) for *E. varleyi*. Artemisa Province: Soroa (JN015142–146); Estación Ecológica Las Terrazas, Sierra del Rosario (JN015147–148; =MNHNCu 1233–1234). Mayabeque Province: Boca de Canasí (JN015149–150; =MNHNCu 1237–1238). Villa Clara Province: Cayo Alto, Reserva Ecológica Sabanas de Santa Clara, Santa Clara (JN015151–152; =MNHNCu 1235–1236). Sancti Spiritus Province: Río Guanayara (JN015153). Camagüey Province: Paso de Lesca, Sierra de Cubitas (JN015154; =MNHNCu 1231). The following are localities and sequence accession numbers (GenBank) for the new species. Camagüey Province: Paso de Lesca, Sierra de Cubitas (JN015155–158; =MNHNCu 1181, 1184, 1185, and 1189). Holguín Province: Loma de la Mensura (JN015159). Granma Province: 1.4 km N Minas del Frío (JN015160); Santo Domingo (JN015161); Marea de Limones, Cabo Cruz (JN015162–165; =MNHNCu 1205, 1206, 1208, 1209). Santiago de Cuba Province: 1.5 km WSW La Tabla (JN015166–173); Gran Piedra (JN015174–177; =MNHNCu 1213–1216). Guantánamo Province: 2.9 km N El Palmar (JN015178–179); 4.7 km N El Palmar (JN015180); 2 km N La Municipión (JN015181); El Salvador (JN015182–185); Santa Rita, Sierra de Canasta (JN015186); Guantánamo City (JN015187–189; =MNHNCu 1220, 1225, and 1226).

Male individuals (n=11) of *E. varleyi* (*sensu lato*) from La Chorrera (Artemisa Province, 22°50'N/82°53'W), 3 individuals (2 males, 1 female) from Soroa (Artemisa Province, 22°47'N/83°00'W) and 18 individuals (12 males, 5 females, 1 juvenile) from Santo Domingo (Granma Province, 20°02'N/76°54'W) were available for cytogenetic analysis. All techniques employed followed the protocols compiled by Schmid *et al.* (2010). Mitotic metaphase chromosomes were prepared according to the bone marrow and the intestinal epithelium suspension techniques. Male meiotic chromosomes in the stage of diakinesis were prepared according to the testis suspension technique. The mitotic chromosomes were conventionally stained, C-banded, and labeled with AgNO₃ or quinacrine mustard. The meiotic chromosomes were conventionally stained. Microscopic analyses were conducted on Zeiss photomicroscopes III, Zeiss fluorescence microscopes, and Zeiss Axiphot microscopes equipped with incident HBO 50W mercury lamp illumination. The various filter combinations necessary for the analyses of metaphases stained with quinacrine mustard were as described by Schmid *et al.* (2010). Photographs were taken on Ortho 25 films (Rollei, Germany) or films with similar properties.

The following abbreviations are used: SVL, snout-vent length; MNHNCu, Museo Nacional de Historia Natural de Cuba; MCZ, Museum of Comparative Zoology at Harvard University; AMNH, American Museum of Natural History; LMD, field number series of Luis M. Díaz (specimens deposited in the MNHNCu); CZACC, zoological collection of the Instituto de Ecología y Sistemática, La Habana; BSC.H, herpetological collection of BIOECO (Centro Oriental de Ecosistemas y Biodiversidad), Museo de Historia Natural Tomás Romay, Santiago de Cuba; MFP, Museo Felipe Poey, Facultad de Biología, Universidad de La Habana. Specimens used for interspecific comparisons are listed in Appendix I.

Eleutherodactylus feichtingeri, new species

Fig. 1A

Holotype. MNHNCu 1181, adult male from the surroundings of Paso de Lesca (21°35'26" N, 77°47'21" W), on the southern slope of Sierra de Cubitas, municipality of Cubitas, Camagüey province, collected by Luis M. Díaz and Antonio Cádiz on August 5, 2010.

Paratypes (n=67). **Males.** Camagüey Province: MNHNCu 1182–1189, with the same data as the holotype, collected by L. M. Díaz, A. Cádiz, and Dariel López; MNHNCu 1190–1191, from Hoyo de Bonet (21°36'N, 77°47'W), Sierra de Cubitas, collected by L. M. Díaz on September 9, 1996; MNHNCu 1192–1193, same locality as the holotype, collected by L. M. Díaz on September 16–19, 2002. Sancti Spiritus Province: MNHNCu 1212, surroundings of Hotel Zaza, Río Zaza, collected by L. M. Díaz on July 11, 1999. Granma Province: MNHNCu 1194–1208, Marea de Limones (19°58'35"N, 77°36'60"W), Niquero, Cabo Cruz, collected by L. M. Díaz and A. Cádiz on October 7, 2010; MNHNCu 1209–1211, same locality, but collected by L. M. Díaz, Freddy Hordies, and André van Hecke, on September 3, 2009; BSC.H 659, 2 km W de Los Muertos, Alegría de Pío, Niquero, collected by Ansel Fong on April 17, 1996. Santiago de Cuba Province: MNHNCu 1213–1217, Gran Piedra (20°00'47"N, 75°37'39"W), Sierra Maestra, 800–900 m a.s.l., Santiago de Cuba, collected by L. M. Díaz and A. Cádiz along the road borders, on October 8, 2010; MNHNCu 1218–1219, surroundings of Santiago de Cuba airport, Santiago de Cuba, collected by L. M. Díaz on May 29, 2000; BSC.H 2476–2480, La Tabla, Tercer Frente, collected by A. Fong and Rolando Viña on April 19, 2000; BSC.H 3176, surroundings of Paso la Mina, 4.5 km S of Cruce de los Baños, Tercer Frente, collected by A. Fong and Rolando Teruel on May 14, 2004; BSC.H 2671–2674, gardens of Hotel Sierramar, Sevilla, Guamá, collected by A. Fong and David Maceira on November 21, 2001; BSC.H 2342–2345, El Olimpo, 4km WNW of Gran Piedra, Santiago de Cuba, collected by A. Fong on February 9, 2000; BSC.H 3156–3157, Providencia, 13.5 km WSW from El Caney, Santiago de Cuba, collected by B. Lauranzón and D. Maceira on April 29, 2004. Guantánamo Province: MNHNCu 1220–1226, City of Guantánamo, crossroad to El Salvador (20°11'17"N, 75°13'40"W), collected by L. M. Díaz and A. Cádiz on October 6, 2010; MNHNCu 330, Arroyón, San Antonio del Sur, collected by Orlando H. Garrido on June 25, 1990; MNHNCu 1227–1229, San Rafael (20° 23'09"N, 74°56'40"W), Yateras, 400 m a.s.l., collected by L. M. Díaz on May 24, 2000. **Female.** Santiago de Cuba Province: BSC.H 3271, 1.2 km NNW of Nuevo Mundo, main road to La Caoba, San Luis, collected by R. Teruel and A. Fong on May 26, 2005.



FIGURE 1. Two cryptic species of Cuban *Eleutherodactylus*: *E. feichtingeri* n. sp. (A), holotype male MNHNCu 1181, 15.2 mm SVL, from Paso de Lesca, Sierra de Cubitas, Camagüey Province; and *E. varleyi* (B), male MNHNCu 1230, 15.5 mm SVL, from the same locality. Photos: L. M. Díaz.

Diagnosis. The new species is a member of the genus *Eleutherodactylus*, subgenus *Euhyas* (*Eleutherodactylus gundlachi* species group, of the *Eleutherodactylus planirostris* species series), based on molecular phylogenetic relationships and morphological similarity to other species, especially *E. varleyi*, following classification of Hedges *et al.* (2008). Males reach 17.4 mm SVL and females 18.5 mm. The new species is similar to *E. varleyi* (Fig. 1B) in the following combination of characters: (1) very small size; (2) partially areolate venter; (3) ventral disc present; (4) dorsolateral rows of enlarged tubercles; (5) a black stripe crossing the supratympanic fold, sur-

rounding a lower highlighted glandular area; (6) external vocal sac; (7) small digital discs; and (8) accentuated polychromatism. Morphologically, the new species differs from *E. varleyi* by having: (1) smaller tympanum [8–15% ($x=12\%$) of head length, vs. 18–26% ($x=21\%$) in *E. varleyi*; Fig. 2]; (2) tympanum is partially or totally pigmented and embedded in the supratympanic stripe which looks like an extended mask, having a sharp contrast with the underlying light stripe (in *E. varleyi*, the tympanum tends to be paler and conspicuous, and mostly interrupts the stripe because of its larger size) (Fig. 3); and (3) different advertisement calls (basically one-note call vs. two-notes call in *E. varleyi*). Both species differ genetically as shown below.

Description. Head as wide as long, its length 32.4–37.7% ($x=34.7\%$) of SVL; snout subacuminate in dorsal view and in profile, slightly overlapping the lower jaw; snout length 36.7–59.2% ($x=41.3\%$) of head length; nostrils rounded, not protuberant, directed laterally, and separated by a distance equivalent to 19.6–29.6% ($x=24.8\%$) of head width; canthus rostralis almost straight in dorsal view; rounded in profile; loreal region gradually sloping to the labial border; lips not flared; interorbital distance 1.2–2.5 ($x=1.7$) times the upper eyelid width, without enlarged tubercles; eyelid skin smooth or with very small granules; loreal area smooth; tympanum superficial, rounded to oval, with poorly distinct annulus, 21.9–42.4% (32.6%) of eye diameter, separated from eye by a distance equivalent to 0.4–1.4 ($x=0.6$) times its own diameter; supratympanic fold distinct; 2–4 postrictal tubercles with glandular features, and very often one of them is very enlarged; choanae 75–100% of third finger disc diameter, oval, partially concealed by palatal shelf of maxillary arch; vomerine odontophores moderate in size, arched, separated from each other by 1/2 times their length, barely reaching the external margins of choanae; tongue sub-oval, its posterior 1/2 not adherent to floor of mouth; external vocal sac simple, hemispherical, extended onto chest.

Dorsal skin with granules and tubercles; two dorsolateral rows of tubercles extend from sacrum to the level of forelimb insertion; many of these tubercles are elongated. Flanks areolate. Supraaxillary, postfemoral, and inguinal glands present, variably evident; inguinal glands commonly highlighted in yellow, yellowish-green, or orange. Venter partially areolate, except on chest and throat; anal opening not extended in sheath; inner part of thigh areolate. Palmar tubercle oval, smooth, 1.5 times longer than thenar tubercle; supernumerary palmar tubercle scarce; subarticular tubercles of fingers oval, not very prominent, and rounded in profile. Finger length order: III > IV > II > I; digital discs small, oval to conical, diameter 33–77% ($x=49.9\%$) of tympanum width. Heels without enlarged tubercles; inner metatarsal tubercle narrow and smooth, 1.5 to 2.5 times longer than the conical outer metatarsal tubercle; supernumerary tubercles flat, scarce, and inconspicuous; subarticular tubercles oval to slightly conical, moderately projected in profile. Toes without defined lateral ridges or basal webbing; circumferential groove bordering the distal half of toe pad; heels touching or barely overlapping each other when flexed legs are held at right angles to sagittal plane; toe length order: IV > III > V > II > I. Hand length 17.2–22.4% ($x=19.3\%$) of SVL; foot length 35.2–45.5% ($x=40.3\%$) of SVL; thigh length 32.8–43.4% ($x=38.2\%$) of SVL; shank length 32.6–44.7% ($x=40.1\%$) of SVL; tarsal length 20.6–28.1% ($x=26.3\%$) of SVL. Measurements of *E. feichtingeri* n. sp. are summarized in Table 1 and compared with those of *E. varleyi*. Only males were included for comparative purposes, considering the still very low number of females available for both species in collections. Holotype measurements are shown independently in Table 1.

Color in life: Coloration is variable; the species is polymorphic and exhibits several patterns. Representative patterns (Fig. 4) are: Pattern A: almost uniformly colored frogs or with faint evidence of suprascapular chevron or middorsal diamond shaped figure. Pattern B: presence of dark chevrons, triangles, or rhomboidal dark blotches, sometimes interconnected to each other; these blotches vary in pigment intensity; lighter areas could be present on head and middorsum. Pattern C: wide dorsal longitudinal light areas; this pattern could be evident as two wide paravertebral stripes (gray, greenish, light brown); but it could give the impression of a single dorsal light area depending on extension and contrast of the dark pigment along the vertebral line. Pattern D: two dorsolateral light stripes, which are usually light orange, gray, greenish tan, or yellowish tan.

The frequencies of the different color patterns are variable among populations. For example, of 18 specimens collected at Marea de Limones (Cabo Cruz), 16 exhibited pattern D and only two displayed pattern C. In the Sierra de Cubitas, of 13 individuals, 7 showed pattern A, 1 had pattern B (including the holotype), 2 had pattern C, and 3 had pattern D. Of 5 individuals from Gran Piedra, only 1 had pattern A, and the others pattern C. Finally, in a sample of seven frogs from the city of Guantánamo (crossroad to El Salvador), 1 had pattern A, 2 had pattern B, 2 had pattern C, and 2 had pattern D.

TABLE 1. Morphological measurements (in mm) of *Eleutherodactylus feichtingeri* n. sp., and *E. varleyi*. Values are means and ranges (in parentheses). Only measurements from adult males are provided.

	<i>E. feichtingeri</i> n. sp. (N=40)	Holotype	<i>E. varleyi</i> (N=40)
Snout-vent length	14.7 (13.3–17.4)	15.2	14.1 (12.2–18.5)
Head length	5.2 (4.6–5.9)	5.4	5.4 (4.6–7.0)
Head width	4.9 (4.4–5.8)	5.3	5.1 (4.2–6.6)
Snout length	2.1 (1.8–3.0)	2.1	2.1 (1.8–3.0)
Upper eyelid width	0.9 (0.8–1.2)	0.8	1.1 (0.8–1.4)
Interocular distance	1.7 (1.3–2.1)	2.1	1.5 (1.2–2.4)
Internarial distance	1.2 (1.0–1.5)	1.3	1.2 (1.0–1.5)
Tympanum width	0.6 (0.4–0.7)	0.7	1.1 (0.8–1.7)
Tympanum height	0.7 (0.3–1.0)	1.0	1.2 (1.0–1.6)
Thigh length	5.6 (4.7–6.5)	6.4	5.8 (4.7–7.5)
Shank length	5.9 (4.9–7.0)	6.0	6.1 (5.5–8.3)
Tarsal length	3.8 (3.1–4.6)	4.0	4.0 (3.4–5.0)
Foot length	5.9 (4.7–7.3)	5.5	5.9 (5.1–8.3)
Hand length	2.8 (2.3–3.5)	2.9	3.0 (2.3–3.9)
Eye-tympanum distance	0.4 (0.3–0.6)	0.3	0.4 (0.1–0.7)
Eye diameter	1.8 (1.6–2.1)	2.0	1.8 (1.4–2.8)
Eye-naris distance	1.2 (1.0–1.5)	1.3	1.4 (1.1–2.0)
Finger disc (III) width	0.3 (0.2–0.4)	0.3	0.3 (0.2–0.4)
Toe disc (IV) width	0.4 (0.3–0.5)	0.3	0.4 (0.2–0.5)

The overall background coloration varies from gray, green, purplish, dark brown, light brown, reddish brown to yellowish brown. Lower flanks are slightly to intensely darker than the dorsum, very often with scattered light areoles. Brown bands or bars on fore- and hindlimbs are well defined, fragmented, or even absent. The supratympanic fold and the tympanic membrane are crossed by a black stripe. The postrictal area is conspicuously white or yellowish green. A half-moon shaped area or a more or less continuous narrow line (usually of the same color as postrictal area) is evident under the eyes. Sides of snout with black pigment, more concentrated along the lower margin of the canthus rostralis. Tip of snout with a vertical pale bar. Venter white or greenish white. Vocal sac usually of same color as belly.

Color in alcohol: Patterns remain after preservation, but most specimens turn gray to greyish brown, with no evidence of green or yellow tones.

Advertisement call description and comparisons. Typical calls of *Eleutherodactylus feichtingeri* n. sp. consist of one short metallic note (Fig. 5A) with a duration of 8–41 milliseconds. In the sonagram, each call usually rises sharply in the first 30–47% of signal duration and then remains constant or lowers slightly in the last part. Maximum amplitude is reached in the first 7–50% ($x=16.6\%$) of call length, after which signal intensity decreases. Some calls show two peaks, the first always more intense than the second. Call duration showed 25.8% of variation within the entire sample. The dominant frequency only varied 4.6%. During a one-hour survey (8 October 2010) along the road from Gran Piedra to the town of Siboney (Santiago de Cuba Province), at an altitudinal gradient from 900 m to sea level (19–26°C), call pattern was the same. Table 2 summarizes call variables for three populations of *E. feichtingeri* from which DNA information is available to support species recognition. In all three cases, the acoustic properties were similar.

TABLE 2. Variation of some acoustic variables in *Eleutherodactylus feichtingeri* n. sp., and *E. varleyi*. Values are means and ranges (in parentheses). Sample sizes are given as N_1 = number of recorded males, and N_2 = total number of analyzed calls. Temperature data were taken during field recordings.

<i>E. feichtingeri</i> n. sp.	Call duration (seconds)	Call rise time (seconds)	Call interval (seconds)	Call rate (calls/minute)	Dominant frequency (kHz)
Sierra de Cubitas $N_1=4$; $N_2=40$ 27.4–27.9°C	0.019 (0.008–0.028)	0.003 (0.002–0.004)	3.0 (1.7–4.7)	27.1 (21.4–37.0)	3.9 (3.7–4.5)
Marea de Limones, Cabo Cruz $N_1=3$; $N_2=30$ 26°C	0.032 (0.021–0.041)	0.004 (0.003–0.006)	2.4 (1.8–3.1)	27.9 (23.0–31.6)	3.7 (3.2–3.9)
Gran Piedra $N_1=4$; $N_2=40$ 20.6°C	0.032 (0.020–0.041)	0.004 (0.002–0.006)	2.0 (1.5–3.1)	32.8 (26.2–35.7)	3.7 (3.5–3.9)
<i>E. varleyi</i>	Call duration + Notes duration (note1/ note 2)	Notes rise time (note1/ note 2)	Call interval	Call rate	Dominant frequency (note 1/ note2)
Sierra de Cubitas $N_1=1$; $N_2=20$ 27.4°C	0.149 (0.135–0.161) 0.028 (0.024–0.030)/ 0.016 (0.011–0.025)	0.008 (0.004–0.011)/ 0.004 (0.003–0.006)	4.5 (3.6–5.6)	34.5	3.5 (3.1–4.3) / 4.1 (3.6–4.5)
Jardín Botánico de Cienfuegos $N_1=3$; $N_2=30$ 23°C	0.210 (0.175–0.271) 0.022 (0.009–0.032)/ 0.015 (0.008–0.022)	0.008 (0.006–0.014)/ 0.003 (0.002–0.008)	4.2 (1.1–6.9)	15.9 (13.6–17.6)	4.5 (3.8–5.5) / 5.6 (4.6–5.9)
Jardín Botánico Nacional, La Habana $N_1=4$; $N_2=40$ 24.6°C	0.193 (0.166–0.250) 0.024 (0.012–0.033)/ 0.015 (0.011–0.020)	0.009 (0.005–0.018)/ 0.004 (0.002–0.007)	2.9 (1.1–6.4)	21.8 (17.9–24.3)	4.5 (3.5–6.5) / 5.4 (4.7–5.9)

In contrast to *Eleutherodactylus feichtingeri* n. sp., calls of *E. varleyi* have two notes and are less intense (not metallic calls). The first note has a duration of 9–32 milliseconds and the second note 8–25 milliseconds, with a note interval of 12–25 milliseconds. The second note tends to be higher pitched than the first note. Sporadically, single notes are given at the beginning of long sequences of typical two-note calls, which are uttered more or less uniformly spaced or in a faster sequence of two or more calls separated from another group by a longer period of time. *Eleutherodactylus varleyi* shows a high variation in the duration, intensity, and pattern of the call. The only individual recorded at Sierra de Cubitas showed a tendency to utter notes with a descending pattern of frequency (Fig. 5B). In a total of 20 calls from this individual, the first note rose in frequency in one call, was sinuous three calls, descended in 12, and had an inverted “U”-shaped pattern in four. The second note of that individual’s calls descended in frequency in 16 calls and rose in four (Fig. 5C–D). In the first note, maximum amplitude was reached at 27–60% ($x=40\%$) of the call length, and in the second note at 12–44% (27%). These differences in the call rise time correspond to signal shape in the oscillogram (Fig. 3C–D). Sympatric individuals of both species were recorded while calling at the same temperature (27.4°C). Table 2 shows call variables for three populations of *E. varleyi*.

Molecular phylogeny. The tree obtained from the Cyt-b sequences (Fig. 6) shows that *Eleutherodactylus feichtingeri* n. sp. and *E. varleyi* form two well-supported clades. DNA analyses also confirm the sympatry of both species in the vicinity of Sierra de Cubitas, Camagüey Province. *Eleutherodactylus varleyi* from Cubitas belongs to a clade of central populations (typical *E. varleyi*) as well as sampled specimens from the Mayabeque Province. However, populations from Artemisa Province (western Cuba) reveal some complex relationships that may indicate the presence of yet another cryptic species, requiring further study. *Eleutherodactylus feichtingeri* shows little genetic variation among populations.

Cytogenetic comparisons. With conventional Giemsa staining, all specimens present a diploid number of $2n = 32$ with two small metacentric chromosomes 1 and 2 and 14 pairs of telo- or submetacentric chromosomes 3–16 of continuously decreasing sizes (Fig. 7). No differences can be found in the conventionally stained karyotypes of both species. C-banding reveals distinct differences in the amount and chromosomal location of the constitutive heterochromatin in the karyotypes of the specimens from La Chorrera and Soroa populations (*Eleutherodactylus varleyi*) in northwestern Cuba and the Santo Domingo population (*E. feichtingeri* n. sp.) in southeastern Cuba. In the karyotypes of *E. feichtingeri* from Santo Domingo the constitutive heterochromatin is located in the centromeric and telomeric regions of all chromosomes. Additionally, distinct interstitial C-bands are present in the larger telocentric chromosome pairs 3–8 (Fig. 8A). In contrast, the constitutive heterochromatin is confined to the centromeric and telomeric regions in the chromosomes of *E. varleyi* from Soroa (Fig. 8B).

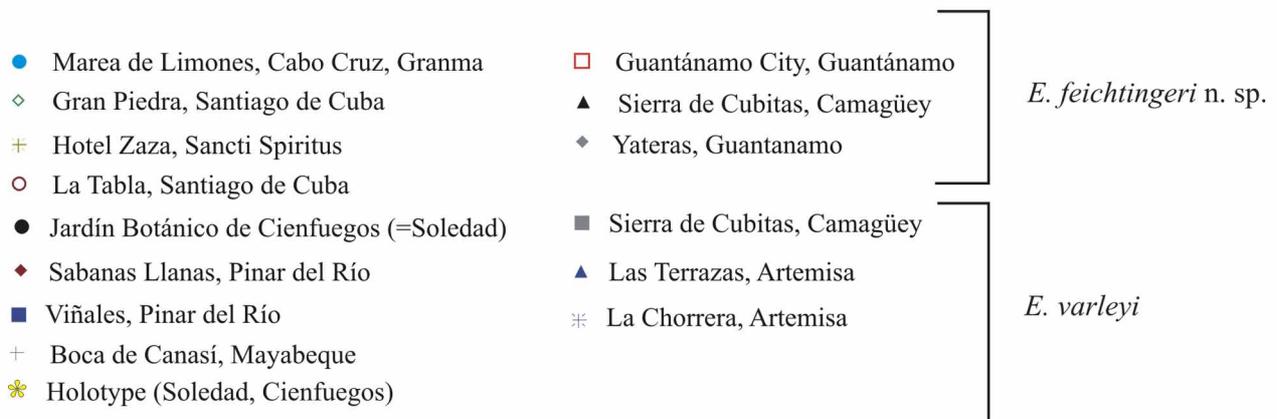
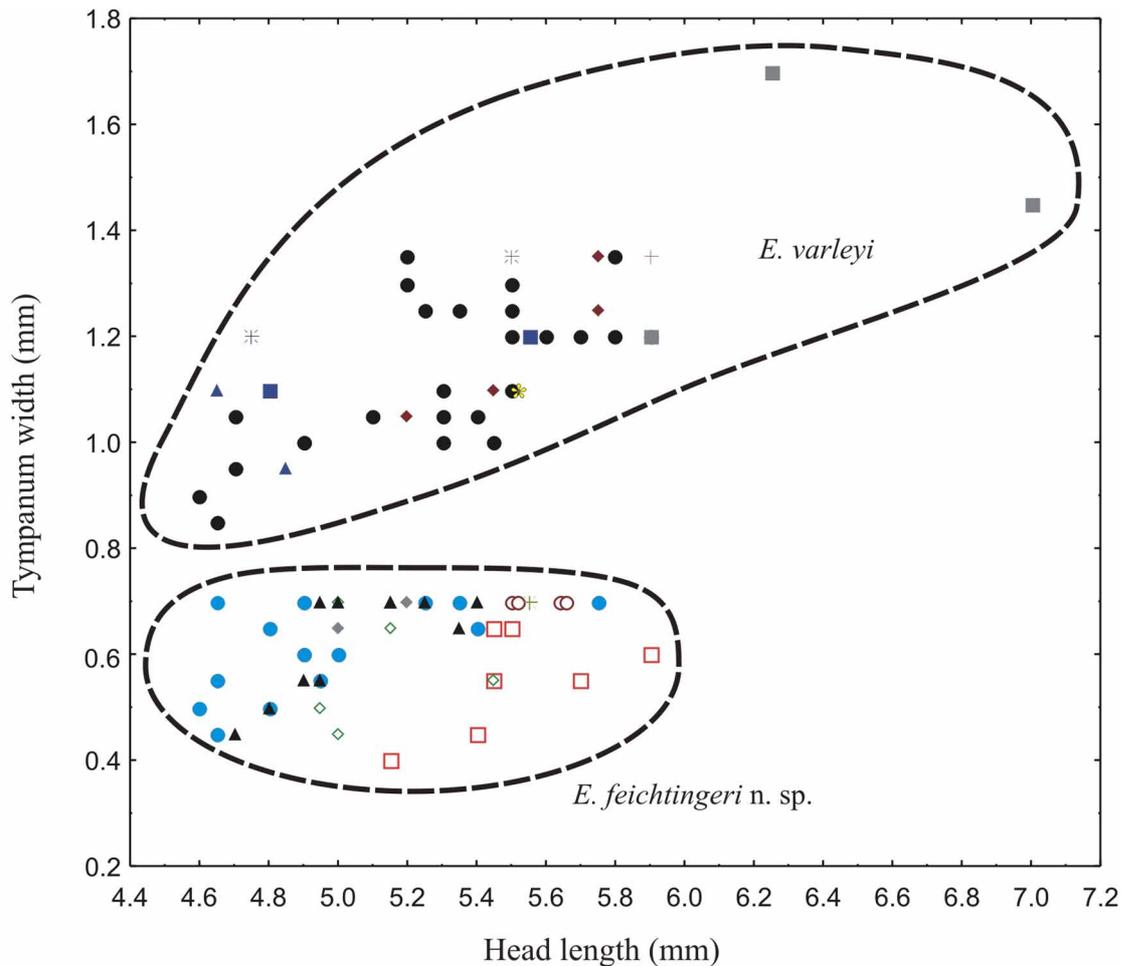


FIGURE 2. Plotting graph of Tympanum width vs. head length in samples (by population) of two closely related species of Cuban *Eleutherodactylus*.

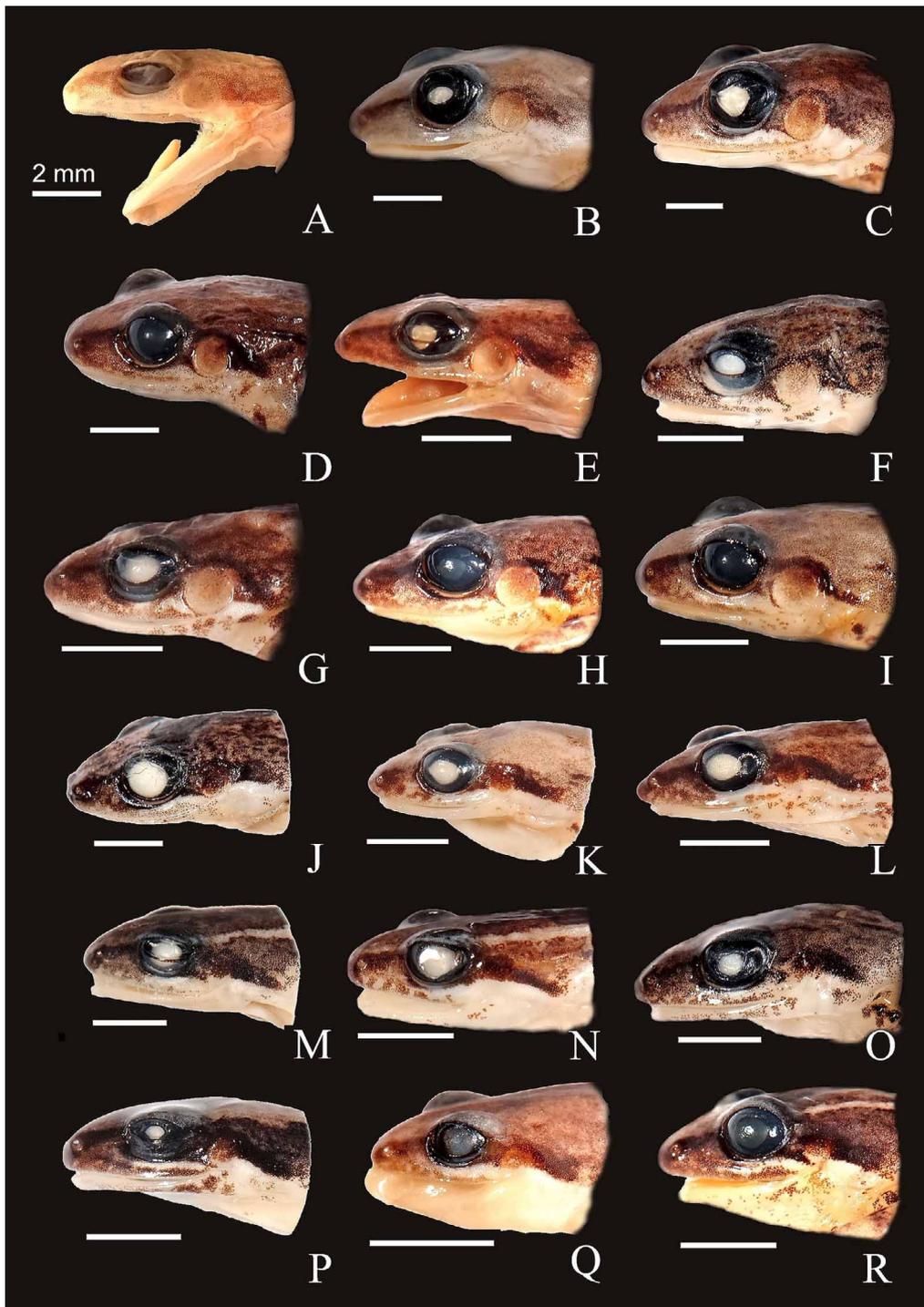


FIGURE 3. Variation in the size of the tympanum and supratympanic stripe in some specimens of *Eleutherodactylus varleyi* (A-I) and *E. feichtingeri* n. sp. (J-R). See Diagnosis to understand the figure. (A) Holotype of *E. varleyi* (MCZ 10601), from Soledad, Cienfuegos Province (only tympanum size is informative, the specimen is discolored); (B) MNHNCu 1231 and (C) MNHNCu 1232, both from Sierra de Cubitas, Camagüey Province; (D) LMD 279, Sabanas Llanas, Alturas de Pizarras del Sur, Pinar del Río Province; (E) MNHNCu 613, Cueva Cheta, Majagua-Canteras, Sierra de San Carlos, Pinar del Río Province; (F) MNHNCu 1235, Campismo La Chorrera, Artemisa Province; (G) Boca de Canasí, Mayabeque Province; (H) LMD 251 and (I) LMD 252, Jardín Botánico de Cienfuegos (=Soledad), Cienfuegos Province; (J) Holotype of *E. feichtingeri* n. sp. (Sierra de Cubitas, Camagüey Province); (K) MNHNCu 1193 and (L) MNHNCu 1192, paratopotypes; (M) MNHNCu 1195 and (N) MNHNCu 1210, Marea de Limones, Cabo Cruz, Granma Province; (O) MNHNCu 1224, Guantánamo City (cross-road to El Salvador), Guantánamo Province; (P) MNHNCu 1217, Gran Piedra, Sierra Maestra, Santiago de Cuba Province; (Q) MNHNCu 1219, Santiago de Cuba City (airport), Santiago de Cuba Province; (R) MNHNCu 1227, San Rafael, Yateras, Guantánamo Province.

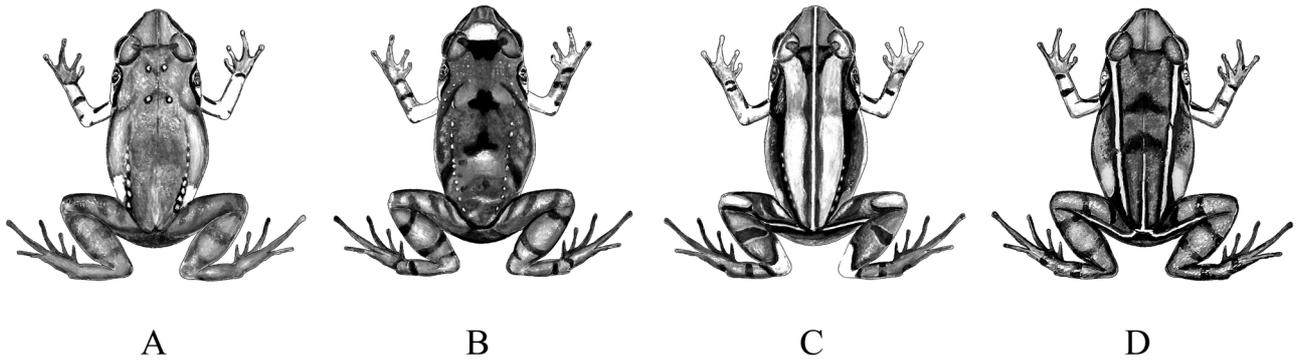


FIGURE 4. Color patterns of *Eleutherodactylus feichtingeri* n. sp. and *E. varleyi* (drawings from Díaz and Cádiz, 2008). See text for pattern descriptions.

Furthermore, the sizes of centromeric C-bands in the chromosomes from *Eleutherodactylus feichtingeri* n. sp. of Santo Domingo are generally larger than those of *E. varleyi* from Soroa. C-banding did not reveal the existence of heteromorphic XY♂/XX♀ or ZZ♂/ZW♀ sex chromosomes in the populations examined.

Silver staining of the nucleolus organizer regions (NORs) reveals that there are two NOR cytotypes in *Eleutherodactylus feichtingeri* n. sp. from Santo Domingo. The more frequent one has the standard NORs in the long-arm paracentromeric region of both telocentric homologues 11 (Fig. 9A). In the other, rarer one, the NOR in one of the homologues 11 is shifted to the short arm by a class II pericentric inversion (Fig. 9B). Individuals homozygous for the inverted NOR have not yet been observed among the low number of examined individuals, but are suspected to occur in this population. Since the NORs in *E. varleyi* from Soroa are also located in the long-arm paracentromeric regions of telocentric homologues 11 (Fig. 9C), the inverted NOR in *E. feichtingeri* is considered to be the derived condition. Several individuals in the Soroa population show a deletion of one of the homologous NORs (Fig. 9C).

In both species, quinacrine staining does not reveal brightly fluorescing heterochromatin in the karyotypes. The chromosomes fluoresce with a uniform intensity over their entire lengths. This observation indicates that the constitutive heterochromatin detected by C-banding is not enriched in AT base pairs (Schmid *et al.*, 2010). Male meiotic chromosomes in the stage of diakinesis show either a ring-like or cross-like pairing arrangement, typical for male meiosis in the highly evolved Anura (Morescalchi, 1971, 1973; Schmid *et al.*, 2010), or else, after terminalization of the chiasmata at the ends of their paired arms, a rod-like or v-shaped end-to-end association.

Distribution. *Eleutherodactylus feichtingeri* n. sp. is widely distributed in part of the central and most of the eastern regions of Cuba (Fig. 10), from sea level to 900 m a.s.l.

Etymology. We take pleasure in naming this species after Dr. Wolfgang Feichtinger from the Biocenter of the University of Würzburg (Germany), for his enthusiastic contributions during field work and improvements in the herpetological work in Cuba.

Natural history. In the Sierra de Cubitas and surrounding plains, *Eleutherodactylus feichtingeri* n. sp. and *E. varleyi* are primarily associated with herbaceous vegetation. Most specimens were collected in an open savanna on serpentine rock, a vegetation complex known as *cuabal* (Fig. 11A). In forested areas of Cubitas, the new species also inhabits the leaf litter and grass stratum of a semideciduous forest on limestone. Through its distributional range, this frog is very common around disturbed habitats, occupying pastures, sugar cane plantations, other agricultural lands, and gardens. In higher mountains, like the Gran Piedra (Sierra Maestra, Santiago de Cuba; ~900 m a.s.l.), *E. feichtingeri* is found in the grass-covered road borders and open herbaceous situations within tropical rain forests.

Males call mainly from grass (Fig. 11B) and vocal activity is more intense after rains. The species has been heard throughout the year at many localities in the mountains of the eastern part of the island, or seasonally (April to December) in the driest lowlands. Sometimes dense choruses occur late in the afternoon (~16:00–19:00 hours). Vocal activity gradually decreases after midnight, and a second peak occurs shortly before dawn. Males call either at a very close distance from each other (0.5–1.0 m) or several meters apart. At Marea de Limones, Cabo Cruz, 16 calling individuals were collected in less than 100 m² at around 20:00 hours (7 October 2010). Vocalizing frogs were found in the following situations: (1) directly on the ground, (2) hidden in the base of grass clumps, (3) exposed at different levels on the grass, (4) on broad leaves of vines (exposed or hidden between two leaves),

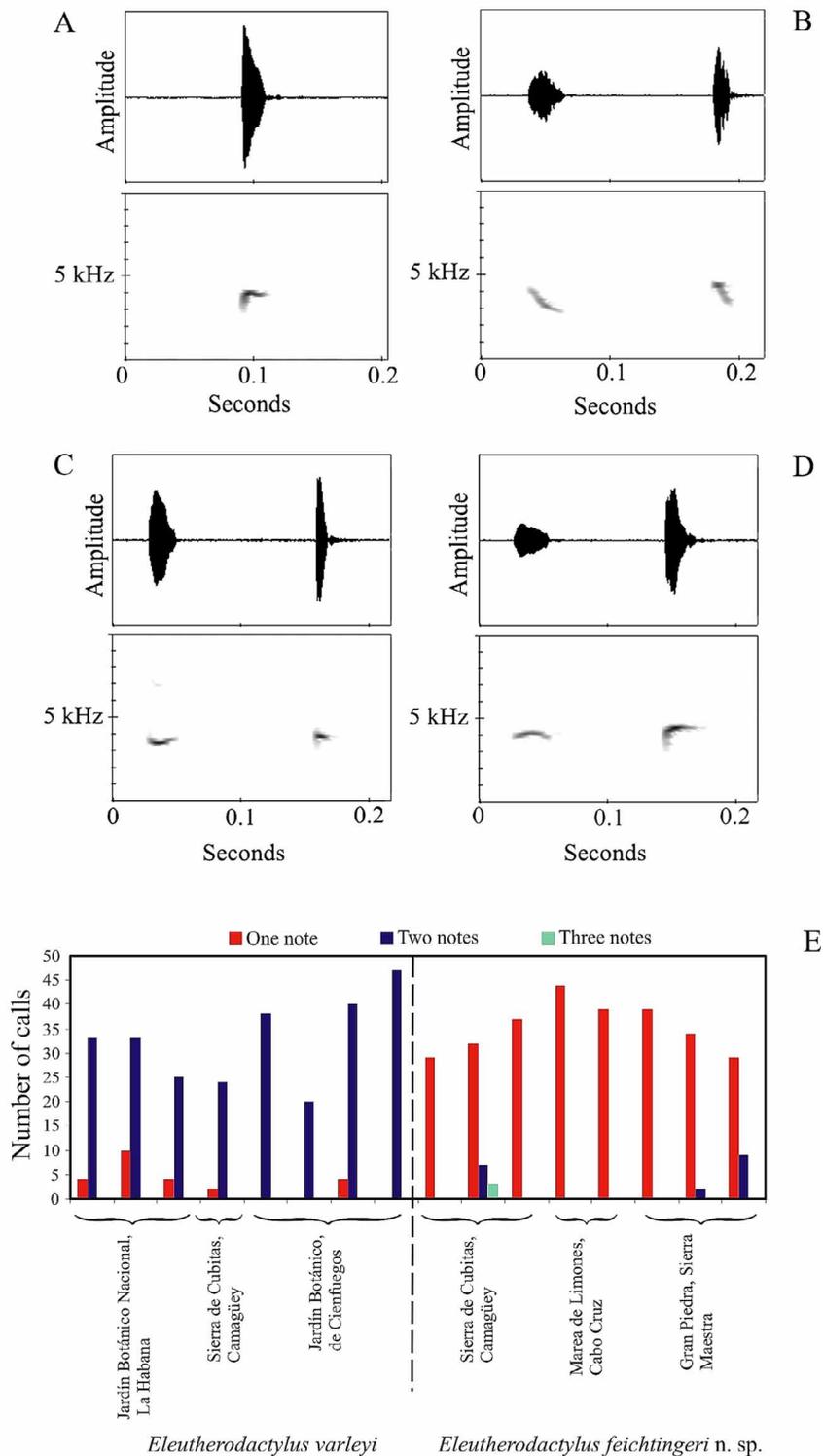


FIGURE 5. Oscillograms and sonagrams (A–D) of advertisement calls of two cryptic species of Cuban *Eleutherodactylus*: *E. feichtingeri* n. sp. (A), from the type locality, voucher male MNHNCu 1181 (holotype), air temperature: 27.4°C, 01:00h, 6Aug.2010; call variation of *E. varleyi* (B–D), same locality, voucher male MNHNCu 1230, 27.4°C, 01:23h, 6Aug.2010. (E) Occurrence of one, two, and three note calls in sound recordings (two minutes long) of male *Eleutherodactylus varleyi* and *E. feichtingeri* n. sp. from the same localities listed in Table 2. Males with shorter periods of recording were not included in the graph. Voucher specimens: *Eleutherodactylus varleyi* (excepting Jardín Botánico Nacional, La Habana): Sierra de Cubitas: MNHNCu 1230; Jardín Botánico de Cienfuegos: LMD 266–269, 22–23°C. *Eleutherodactylus feichtingeri* n. sp.: Sierra de Cubitas: MNHNCu 1181, 1184, and 1189, 27.4–27.9°C; Marea de Limones, Cabo Cruz: MNHNCu 1208 and 1209, 26°C; Gran Piedra: MNHNCu 1213, 1214, and 1216, 20.6°C



FIGURE 6. Phylogenetic tree of Cuban grass frogs, *Eleutherodactylus varleyi* and its close relative *E. feichtingeri* n. sp. The tree was constructed from DNA sequences of the mitochondrial Cyt-b gene, using maximum likelihood. Bootstrap support values are indicated on nodes. In the tree, the holotype of *E. feichtingeri* is GenBank JN015155.

(5) on small bush leaves 0.2–1m high, or (6) inside a rolled leaf on the ground of the forest. Typically, males call in a horizontal position. Specimen MNHNCu 1226 contains a large encysted nematode between the arm and the chest cavity, measuring 17.3 mm when distended (frog SVL = 16.4 mm).

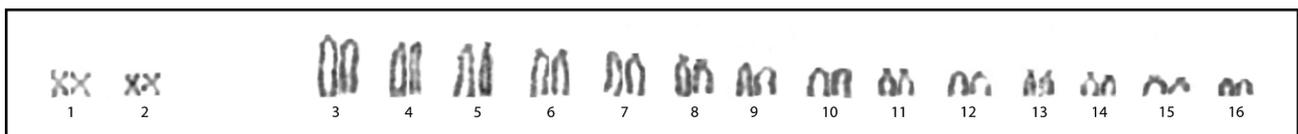


FIGURE 7. Conventionally (Giemsa) stained karyotype of a male specimen of *Eleutherodactylus varleyi* collected in Soroa, Sierra del Rosario (Artemisa Province). Note the two small metacentric chromosomes 1 and 2 and the 14 telo- or subtelocentric chromosomes 3–16 of continuously decreasing sizes.

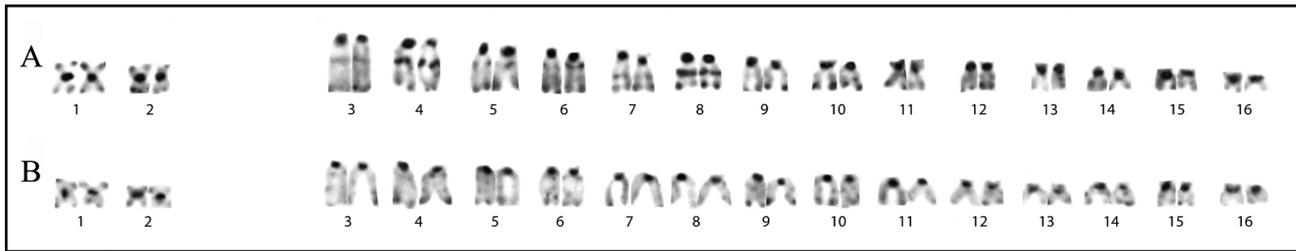


FIGURE 8. C-banded karyotypes of (A) a male of *Eleutherodactylus feichtingeri* n. sp. specimen from Santo Domingo, Sierra Maestra (Granma Province) and (B) a female specimen of *E. varleyi* from Soroa, Sierra del Rosario (Artemisa Province). Note the distinct interstitial C-bands in the larger telocentric chromosome pairs 3–8 in the karyotype of the individual from the Santo Domingo population.

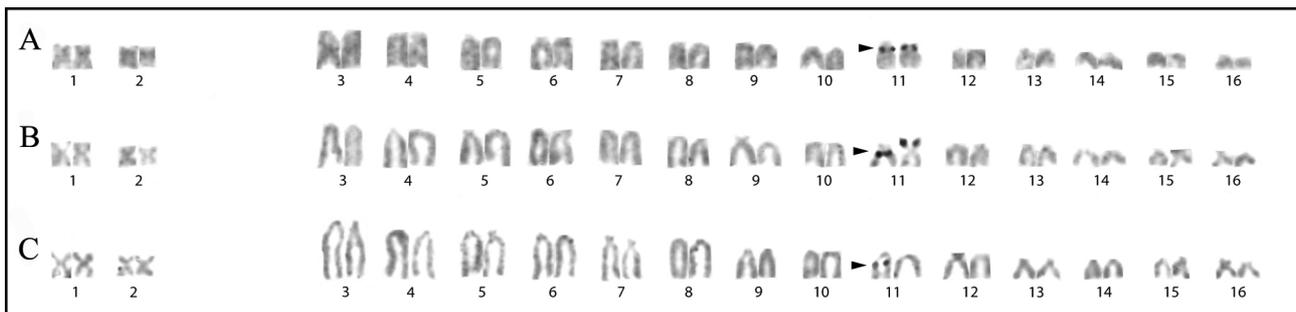


FIGURE 9. Silver-stained nucleolus organizer regions (NORs) in the karyotypes of (A) a male and (B) a female specimen of *Eleutherodactylus feichtingeri* n. sp. collected in Santo Domingo, Sierra Maestra (Granma Province), as well as (C) a male specimen of *E. varleyi* from Soroa, Sierra del Rosario (Artemisa Province). Note the two NOR cytotypes in the Santo Domingo population and the deletion of one of the homologous NORs in the karyotype of the specimen from the Soroa population.

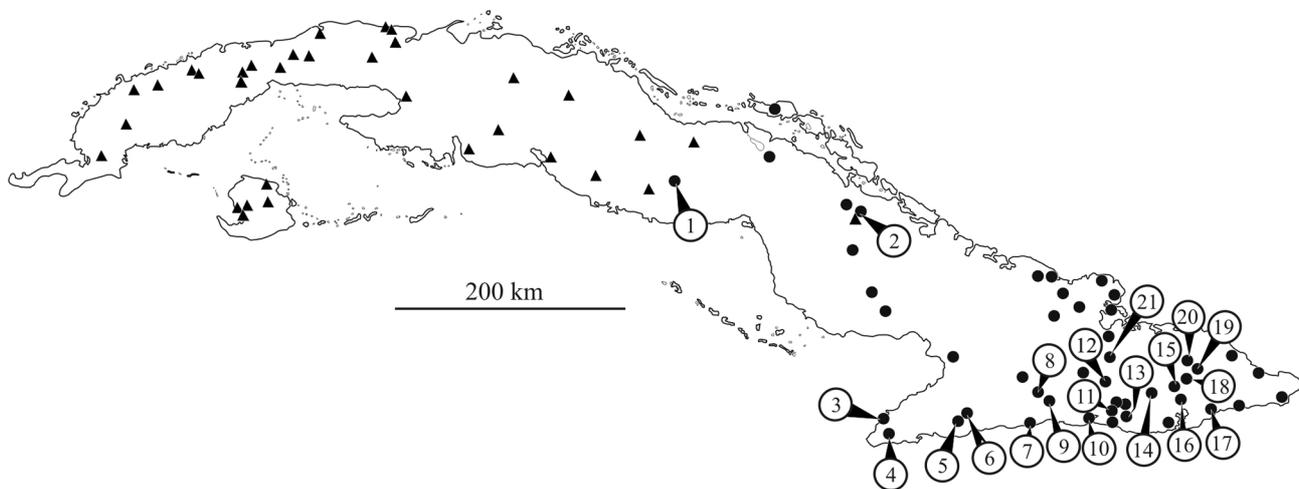


FIGURE 10. Distribution of *E. varleyi* (triangles) and *E. feichtingeri* n. sp. (circles). Numbered localities are those where type specimens and tissue samples were collected; those not numbered are acoustic records: (1) surroundings of Hotel Zaza, Sancti Spiritus Province; (2) Paso de Lesca, Sierra de Cubitas, Camagüey Province; (3) Marea de Limones, Cabo Cruz, Granma Province; (4) Los Muertos, Alegría de Pío, Niquero, Granma Province; (5) Minas del Frío, Sierra Maestra, Granma Province; (6) Santo Domingo, Sierra Maestra Granma Province; (7) Hotel Sierramar, Sevilla, Guamá, Santiago de Cuba Province; (8) La Tabla, Sierra Maestra, Santiago de Cuba Province; (9) 4.5 km S Cruce de los Baños, Tercer Frente, Santiago de Cuba Province; (10) Ciudad de Santiago de Cuba, Santiago de Cuba Province; (11) Providencia, 13 km WNW of El Caney, Santiago de Cuba Province; (12) 1.2 km NNW of Nuevo Mundo, mainroad to La Caoba, San Luis, Santiago de Cuba Province; (13) Gran Piedra, Sierra Maestra, Santiago de Cuba Province; (14) Santa Rita, Guantánamo Province; (15) El Salvador, Guantánamo Province; (16) City of Guantánamo, Guantánamo Province; (17) Arroyón, San Antonio del Sur, Guantánamo Province; (18) El Palmar, Guantánamo Province; (19) San Rafael, Yateras, Guantánamo Province; (20) La Munición, Guantánamo Province; (21) Loma de La Mensura, Holguín Province.



FIGURE 11. Sierra de Cubitas and surrounding plains (A), Camagüey Province, where *Eleutherodactylus feichtingeri* n. sp. and *E. varleyi* are primarily associated with herbaceous vegetation. Calling male (B) of *E. feichtingeri* from Marea de Limones, Cabo Cruz, Granma Province; note the distended vocal sac. Photos: L. M. Díaz.

Discussion

Within the *Eleutherodactylus gundlachi* species Group (*sensu* Hedges *et al.*, 2008), *E. gundlachi* is morphologically the most divergent species. This species reaches the largest size (to 25 mm SVL) in the group, has longer hind limbs, darker overall coloration, lacks a black supratympanic fold surrounding a lower light area, has a suprascapular W-shaped fold and a conspicuously pustulose body with prominent glandular areas, granular belly, and vividly orange-red flash areas on thighs and groins. *Eleutherodactylus intermedius* and *E. tetajulia*, a pair of closely related species (as first considered by Estrada and Hedges, 1996), have stocky bodies, short limbs and digits, dorsum smooth or with series of small tubercles, robust heads, and belly with brown reticulations. These two species inhabit the leaf litter of the mountain forests of Eastern Cuba, and occur sympatrically (but not syntopically, as yet known) with *E. feichtingeri* n. sp. in some localities. *Eleutherodactylus adelus* was considered related to *E. varleyi* (*sensu lato*) by Díaz *et al.* (2003) based on many shared external characters, and both species occur together in the pine forests of Alturas de Pizarras del Sur (Pinar del Río Province). From *E. varleyi* and *E. feichtingeri*, *E. adelus* differs by a characteristic combination of black and pale stripes, a slightly more stylized body, presence of paravertebral folds at middorsum, different anatomy of the hyoid plate, and distinctive vocalizations that are broadcast at opposite times of the day (Díaz *et al.*, 2003).

Eleutherodactylus varleyi and *E. feichtingeri* n. sp. may show character displacement in the area of sympatry, in both morphology and vocalization. Three males of *E. varleyi* collected in the vicinity of Sierra de Cubitas show differences from allopatric populations of that species and from *E. feichtingeri*. They are larger (15.5–18.5 mm SVL; the largest males that were examined during the present study) than those of *E. feichtingeri*, with longer heads (length 37–38% of SVL vs. 33–36% in *E. feichtingeri* from Cubitas; width 35–39% of SVL vs. 32–36% in *E. feichtingeri*) and with a more evident indentation behind the head (not pronounced in *E. feichtingeri*). The three individuals were almost uniformly colored.

A direct reference to the call patterns of *E. feichtingeri* n. sp. and *E. varleyi* is provided in the supplementary CD of Díaz and Cádiz (2008). Recordings of the new species' typical advertisement calls from Gran Piedra (Santiago de Cuba) are referred as “call pattern II of *E. varleyi*”. Two-note calls and multinote emissions were suggested to be territorial calls of this call pattern by Díaz *et al.* (2003) and are sporadically emitted; a recording of these calls from the surroundings of Hotel Zaza, Sancti Spiritus Province (voucher paratype MNHNCu 1212), is also available in Díaz and Cádiz (2008). Advertisement calls of *E. varleyi* show more variation in temporal and spectral properties than the analyzed populations of *E. feichtingeri*. Díaz *et al.* (2003) reported four common call patterns in the first note of *E. varleyi*, and a usual ascendant pattern of frequency in the second note. More recently, Cádiz (2008) found six different spectral patterns in the first note and eight for the second note in calling males from La Habana, and the common combination was an ascendant pattern in both notes. The tendency to have a

descending pattern of frequency modulation in *E. varleyi* from Cubitas was not observed in other populations and may be another example of character displacement. Additional sonagrams and oscillograms were presented by Díaz *et al.* (2003) and Díaz and Cádiz (2008).

The discovery of *E. feichtingeri* **n. sp.** demonstrates that new species of frogs continue to be found in Cuba. Recently, Rodríguez *et al.* (2009) analyzed the genetic variation of *E. auriculatus* (subgenus *Eleutherodactylus*; *E. auriculatus* species group) suggesting the existence of at least four new cryptic species that still need to be named and described. These species also differ in their advertisement calls (Alonso *et al.*, 2007; Díaz and Cádiz, 2008). Additional new species have been identified by two of the authors (LMD and SBH).

The karyotype ($2n = 32$) of *Eleutherodactylus feichtingeri* and *E. varleyi* is common among members of the *Eleutherodactylus (Euhyas) planirostris* species series, *Eleutherodactylus (Euhyas) zugii* species series, *Eleutherodactylus (Euhyas) armstrongi* species series and *Eleutherodactylus (Euhyas) luteolus* species series. Furthermore, this karyotype is considered to be basic in the *Eleutherodactylus (Pelorius) ruthae* species series, *Eleutherodactylus (Pelorius) inoptatus* species series, *Eleutherodactylus (Syrrhophus) symingtoni* species series, and *Eleutherodactylus (Syrrhophus) longipes* species series (Schmid *et al.*, 2010).

Dissimilarities in the amount, chromosomal location, and DNA base pair composition of the constitutive heterochromatin in the karyotypes of phylogenetically closely related terraranan species can assist with the unambiguous identification of species or with the discovery of cryptic species, as is the case of *E. feichtingeri* **n. sp.** and *E. varleyi*. For example, Kaiser *et al.* (1995) showed that the otherwise identical karyotypes of the closely related *Pristimantis terraebolivaris* from northern Venezuela and *P. charlottevillensis* from Tobago, both belonging to the *Pristimantis (Pristimantis) conspicillatus* species group, can be differentiated by the amount of centromeric heterochromatin and the chromosomal location of the NORs. *Eleutherodactylus blairhedgesi* and *E. thomasi zayasi* are closely related taxa of the *Eleutherodactylus (Euhyas) pinarensis* species group. Both taxa possess the same diploid chromosome number and morphology of chromosomes, have very similar C-band patterns, and the same chromosomal locations of the NORs, but they are distinguished by mithramycin fluorescence staining. In *E. thomasi zayasi*, mithramycin-positive (GC pair rich) paracentromeric heterochromatic bands are located in the long arms of chromosomes 1 and 2, and a mithramycin-negative paracentromeric heterochromatic band (GC base pair depauperate) is present in the long arms of chromosome pair 3, which all are absent in the karyotype of *E. blairhedgesi* (Schmid *et al.* 2010). *Eleutherodactylus bresslerae* and *E. michaelschmidi* are allopatric sister species within the *Eleutherodactylus (Euhyas) ricordii* species group (Díaz *et al.*, 2007). Both species have the same chromosome number and chromosome morphology, very similar C-band patterns, and identical location of NORs. However, both species can be clearly distinguished on the basis of quinacrine staining. Whereas all chromosomes of *E. bresslerae* exhibit a uniform fluorescence without any brightly fluorescing Q^+ heterochromatin, in *E. michaelschmidi* the telomeric heterochromatin in the long arms of chromosome pairs 7, 9, and 11 is distinguished by extremely bright Q^+ fluorescence (Díaz *et al.*, 2007; Schmid *et al.* 2010).

Interspecific comparisons of C-band patterns have been important for cytotaxonomic studies. It has been shown that, although many species of the various amphibian genera have a shared similarity in chromosome number and morphology, there can be extensive differences in the position and amount of heterochromatin (King, 1980; Schmid, 1978, 1980; Schmid *et al.*, 1990; Nardi *et al.*, 1986; Sessions and Wiley, 1985; Green and Sessions, 2007). An evolutionary relationship between the heterochromatic patterns in amphibian chromosomes could not be established, except for the origin of heteromorphic sex chromosomes and some supernumerary B chromosomes. Evidently, chromosomal locations and amounts of heterochromatic regions are variable factors that change rapidly in the course of evolution (John, 1988). As emphasized by King (1990), C-band patterns have significantly contributed to the differentiation of karyotypes between related species, but observed differences in the position and content of constitutive heterochromatin between amphibian karyotypes show that no two species in the most related taxa which have been studied have identical C-band patterns. Thus, comparison of species of the urodelan genera *Triturus* (Macgregor and Sessions, 1986) and *Hydromantes* (Nardi *et al.*, 1986), as well as the anuran genera *Litoria* (King, 1980; Schmid *et al.*, in preparation), *Bufo* (Schmid, 1978; Matsui *et al.*, 1985; Miura, 1995), *Gastrotheca* and *Neobatrachus* (Schmid *et al.*, in preparation) demonstrate striking interspecific C-band variation in species with essentially the same karyotype.

C-band-positive constitutive heterochromatin is characterized by containing highly repetitive DNA sequences. So far, repetitive DNA has no known genetic function. Hence, sequence changes within reiterated DNA can be considered neutral. Since they are not subjected to evolutionary selection pressure, sequence changes in repetitive

DNAs can be accumulated and fixed in genomes much faster than can the changes in functional (transcribed) DNA. As a consequence, constitutive heterochromatin is heterogeneous within and between species (Schmid *et al.*, 2010).

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APPENDIX I. Specimens examined for comparisons

Note: Two Cuban provinces (Artemisa and Mayabeque) were officially recognized in 2010 after division of the province formerly known as La Habana, and incorporation (in Artemisa) of a portion of the province of Pinar del Río. The province Ciudad de La Habana is now properly La Habana.

Eleutherodactylus varleyi (N=119).— **Pinar del Río Province:** LMD 231, 232, 240–245, Loma del Espejo, Alturas de Pizarras del Sur, km 41 Carretera de Luis Lazo; MNHNCu 613, Cueva Cheta, Majagua Canteras; LMD 432, Mil Cumbres, Sierra del Rosario; AMNH 59834, 4.4 mi NW San Vicente; AMNH 61937–44, 5.6 mi NW San Vicente. **Artemisa Province:** MNHNCu 1233–1234, and MFP 11521–11523, Estación Ecológica Las Terrazas, Sierra del Rosario; MNHNCu 1239, Río Bayate, Reserva de la Biosfera Sierra del Rosario, Sierra del Rosario. MNHNCu 1235–1236, Campismo La Chorrera,

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Artemisa. **La Habana Province:** CZACC jar 309 (a single specimen without number), Atabey, Playa. **Mayabeque Province:** MNHNCu 250, Madruga; LMD 276–278, Peñas Blancas; MNHNCu 1237–1238, Boca de Canasí, Santa Cruz del Norte. **Isla de la Juventud:** CZACC jar 311 (18 specimens without individual numbers), Hotel Colony, Siguanea; AMNHN 63292–63308, Nueva Gerona. **Villa Clara Province:** LMD 247–248, Placetas; AMNH 61950, 8 mi S Manicargua; MNHNCu 1235–1236, Cayo Alto, Reserva Ecológica Sabanas de Santa Clara, Santa Clara. **Cienfuegos Province:** LMD 251–269, Jardín Botánico de Cienfuegos; MCZ 10601 (Holotype), Soledad (=Jardín Botánico de Cienfuegos); AMNH 92430–92453, Soledad (=Jardín Botánico de Cienfuegos). **Camagüey Province:** MNHNCu 1230–1232, Paso de Lesca, Sierra de Cubitas.

Eleutherodactylus phyzelus (= *E. varleyi*).— AMNH 59832 (Holotype), 4.4 miles NE of San Vicente, Pinar del Río Province.

Eleutherodactylus adelus.— MNHNCu 874 (Holotype), Loma del Espejo, Alturas de Pizarras del Sur, km 41 Carretera de Luis Lazo, Pinar del Río Province.